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(71) Applicant: **TAKARA SHUZO CO. LTD.**
Kyoto-shi, Kyoto 612 (JP)

(72) Inventors:
- **TAKAKURA, Hikaru**
  Otsu-shi, Shiga 520-22 (JP)
- **MORISHITA, Mio**
  Otsu-shi, Shiga 520 (JP)
- **YAMAMOTO, Katsuhiko**
  Otsu-shi, Shiga 520-21 (JP)
- **MITTA, Masanori**
  Kyotanabe-shi, Kyoto 610-03 (JP)
- **ASADA, Kiyozo**
  Koka-gun, Shiga 520-33 (JP)
- **TSUNASAWA, Susumu**
  Otsu-shi, Shiga 520-21 (JP)
- **KATO, Ikunoshin**
  Uji-shi, Kyoto 611 (JP)

(74) Representative:
Vossius, Volker, Dr. et al
Dr. Volker Vossius,
Patentanwaltskanzlei - Rechtsanwaltskanzlei,
Holbeinstrasse 5
81679 München (DE)

(54) ULTRATHERMOSTABLE PROTEASE GENES

(57) There are provided hyperthermostable proteases having an amino acid sequences represented by SEQ ID Nos. 1, 3 and 5 of the Sequence Listing or functional equivalents thereof and hyperthermostable protease genes encoding those hyperthermostable protease. There is also disclosed a process for preparation of a hyperthermostable protease by culturing a transformant containing the gene.

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Description

TECHNICAL FIELD

The present invention relates to a hyperthermostable protease useful as an industrial enzyme, a gene encoding the same and a method for preparation of the enzyme by the genetic engineering.

BACKGROUND ART

The proteases are the enzymes which cleave peptide bonds in the proteins, and a number of the proteases have been found in animals, plants and microorganisms. They are used not only as reagents for research works and medical supplies, but also in industrial fields such as additives for detergents, food processing and chemical synthesis utilizing the reverse reactions, and it can be said that they are very important enzymes from an industrial viewpoint. For proteases to be used in industrial fields, since very high physical and chemical stabilities are required, in particular, enzymes having high thermostabilities are preferred to use. At present, proteases predominantly used in industrial fields are those produced by bacteria of the genus Bacillus because they have relatively high thermostability.

However, enzymes having further superior properties are desired and activities have been attempted to obtain enzymes from microorganisms which grow at high temperature, for example, thermophiles of the genus Bacillus.

On the other hand, a group of microorganisms, named as hyperthermophiles, are well adapted themselves to high temperature environments and therefore they are expected to be a source supplying various thermostable enzymes. It has been known that one of these hyperthermophiles, Pyrococcus furiosus, produces proteases [Appl. Environ. Microbiol., volume 56, page 1992-1998 (1990), FEMS Microbiol. Letters, volume 71, page 17-20 (1990), J. Gen. Microbiol., volume 137, page 1193-1199 (1991)].

A hyperthermophile belonging to the genus Pyrococcus, Pyrococcus sp. Strain KOD1 is reported to produce a thiol protease (cysteine protease) [Appl. Environ. Microbiol., volume 60, page 4559-4566 (1994)]. Bacteria belonging to the genus Thermococcus, Staphylothermus and Thermobacteroides, which are also hyperthermophiles, are known to produce a protease [Appl. Microbiol. Biotechnol., volume 34, page 715-719 (1991)].

OBJECTS OF THE INVENTION

As the proteases produced by these hyperthermophiles have high thermostabilities, they are expected to be applicable to new applications to which any known enzymes has not been utilized. However, the above publication merely teach that thermostable protease activities present in cell-free extract or crude enzyme solution obtained from culture supernatant, and there is no disclosure about properties of isolated and purified enzymes and the like. Only a protease produced by strain KOD1 is obtained as the purified form. However, since a cysteine protease has the defect that it easily loses the activity by oxidation, it is disadvantageous in the industrial use. In addition, since a cultivation of microorganisms at high temperature is required to obtain enzymes from these hyperthermophiles, there is a problem in industrial production of the enzymes.

In order to solve the above problems, an object of the present invention is to provide a protease of the hyperthermophiles which is advantageous in the industrial use, to isolate a gene encoding a protease of the hyperthermophiles, and to provide a method for preparation of a hyperthermostable protease using the gene by the genetic engineering.

DISCLOSURE OF THE INVENTION

In order to obtain a hyperthermostable protease gene, the present inventors originally tried to purify a protease from microbial cells and a culture supernatant of Pyrococcus furiosus DSM3638 so as to determine a partial amino acid sequence of the enzyme. However, purification of the protease was very difficult in either cases of using the microbial cells or the culture supernatant, and the present inventors failed to obtain such an enzyme sample having sufficient purity for determination of its partial amino acid sequence.

As a method for cloning a gene for an objective enzyme without any information about a primary structure of the enzyme protein, there is an expression cloning method. For example, a pullulanase gene originating in Pyrococcus woesei (WO92/02614) has been obtained according to this method. However, in an expression cloning method, a plasmid vector is generally used and, in such case, it is necessary to use restriction enzymes which can cleave an objective gene into relatively small DNA fragments so that the fragments can be inserted into the plasmid vector without cleavage of any internal portion of the objective gene. Therefore, the expression cloning method is not always applicable to cloning of all kind of enzyme genes. Furthermore, it is necessary to test for an enzyme activity of a large number of clones and this operation is complicated.

The present inventors have attempted to isolate a protease gene by using a cosmid vector which can maintain a

larger DNA fragment (30-50kb) instead of a plasmid vector to prepare a cosmid library of Pyrococcus furiosus genome and investigating cosmid clone in the library to find out a clone expressing a protease activity. By using the cosmid vector, the number of transformants to be screened can be reduced in addition to lowering of possibilities of cleavage of a internal portion of the enzyme gene. On the other hand, since the copy number of a cosmid vector in a host cell is not higher than that of a plasmid vector, it may be that an amount of the enzyme expressed is too small to detect it.

In view of high thermostability of the objective enzyme, firstly, the present inventors have cultured respective transformants in a cosmid library, separately, and have combined this step with a step for preparing lysates containing only thermostable proteins from the microbial cells thus obtained, and used these lysates for detecting the enzyme activity. Further, the use of the gelatin-containing SDS-polyacrylamide gel electrophoresis for detecting the protease activity allowed the detection of a trace amount of the enzyme activity.

Thus, the present inventors obtained several cosmid clones expressing the protease activity from the cosmid library of Pyrococcus furiosus and successfully isolated a gene encoding a protease from the inserted DNA fragment contained in the clones. In addition, the present inventors confirmed that a protease encoded by the gene has the extremely high thermostability.

By comparing an amino acid sequence of the hyperthermostable protease deduced from the nucleotide sequence of the gene with amino acid sequences of known proteases originating in microorganisms, homology of the amino acid sequence of the front half portion of the hyperthermostable protease with those of a group of alkaline serine proteases, a representative of which is subtilisin, has been shown. In particular, the extremely high homology has been found at each region around the four amino acid residues which are known to be important for the catalytic activity of the enzyme. Thus, since the protease produced by Pyrococcus furiosus, which is active at such a high temperature that proteases originating in mesophiles are readily inactivated, has been shown to retain a structure similar to those of enzymes from mesophiles, it has been suggested that similar proteases would also be produced by hyperthermophiles other than Pyrococcus furiosus.

Then, the present inventors have noted possibilities that, in the nucleotide sequence of the hyperthermostable protease gene obtained, the nucleotide sequence encoding regions showing high homology with subtilisin and the like can be used as a probe for detecting hyperthermostable protease gene, and have attempted to detect protease genes originating in hyperthermophiles by PCR using synthetic DNAs designed based on the nucleotide sequences as primers so as to clone the gene. As a result, it was found that a fragment of gene having the homology with the above gene existed in a hyperthermophile, Thermococcus celer DSM2476. The cloning of the full length of the gene was difficult and this was thought to be due to that the product derived from the gene was harmful to the host.

The present inventors used Bacillus subtilis as a host for cloning and found that harbouring of the full length gene was possible and the expressed protease was extracellularly secreted, further revealed that the expressed protease showed the protease activity at 95 °C and had the high thermostability. Upon this, the molecular weight of a protease encoded by the gene was found to be less than half of that of the high-molecular protease derived from the Pyrococcus furiosus described above.

In addition, by hybridization using a fragment of the gene as a probe, we found that the second protease gene different from that of the high-molecular protease was present in Pyrococcus furiosus. The gene encodes a protease having a similar molecular weight to that of the hyperthermostable protease derived from Thermococcus celer, and the gene was isolated and introduced into Bacillus subtilis and, thereby, a product expressed from the gene was extracellularly secreted. The expressed protease showed the enzyme activity at 95 °C and had the high thermostability. In addition, the amino acid sequence of a mature protease produced by processing of the protease was revealed.

As these two kinds of proteases are extracellularly secreted without any special procedures, it is thought that a signal peptide encoded by the gene itself functions in Bacillus subtilis. The amount of expressed both proteases per culture is remarkably higher as compared with the high-molecular protease derived from Pyrococcus furiosus which is expressed in Escherichia coli or Bacillus subtilis. In addition, when the gene is expressed by utilizing a promoter of the subtilisin gene and a signal sequence, the amount of the expressed protease was further increased.

Furthermore, the present inventors prepared a hybrid gene encoding a hybrid protease, i.e., a fusion protein from both proteases, and confirmed that an enzyme expressed by said hybrid gene showed protease activity at high temperature like the above hyperthermostable protease.

SUMMARY OF THE INVENTION

The first aspect of the present invention provides a hyperthermostable protease having the amino acid sequence described in SEQ ID No. 1 of the Sequence Listing or functional equivalents thereof as well as a hyperthermostable protease gene encoding the hyperthermostable proteases, inter alia, a hyperthermostable protease gene having the nucleotide sequence described in SEQ ID No. 2 of the Sequence Listing. Further, a gene which hybridizes with this hyperthermostable protease gene and encodes a hyperthermostable protease is also provided.

In addition, the second aspect of the present invention provides a hyperthermostable protease having the amino

acid sequence described in SEQ ID No. 3 of the Sequence Listing or functional equivalents thereof as well as a hyperthermostable protease gene encoding the hyperthermostable proteases, inter alia, a hyperthermostable protease gene having the nucleotide sequence described in SEQ ID No. 4 of the Sequence Listing. Further, a gene which hybridizes with this hyperthermostable protease gene and encodes a hyperthermostable protease is also provided.

In addition, the third aspect of the present invention provides a hyperthermostable protease having the amino acid sequence described in SEQ ID No. 5 of the Sequence Listing or functional equivalents thereof as well as a hyperthermostable protease gene encoding the hyperthermostable proteases, inter alia, a hyperthermostable protease gene having the nucleotide sequence described in SEQ ID No. 6 of the Sequence Listing. Further, a gene which hybridizes with this hyperthermostable protease gene and encodes a hyperthermostable protease is also provided.

Further, the present invention provides a method for preparation of the hyperthermostable protease which comprises cultivating a transformant containing the hyperthermostable protease gene of the present invention, and collecting the hyperthermostable protease from the culture.

As used herein, the term "functional equivalents" means as follows:

It is known that although, among naturally-occurring proteins, a mutation such as a deletion, an addition, a substitution and the like of one or a few (for example, up to 5% of the whole amino acids) amino acid(s) can occur in the amino acid sequence thereof due to the modification reaction and the like of the produced proteins in the living body or during purification besides the polymorphism or mutation of the genes encoding them, there are proteins, in spite of the mutation described above, showing a substantially equivalent physiological or biological activity to that of the proteins having no mutation. When the proteins have the slight difference in the structures and, nevertheless, the great difference in the functions thereof is not recognized, they are called functional equivalents. This is true when the above mutations are artificially introduced into the amino acid sequence of the proteins and, in this case, further a more variety of mutants can be made. For example, a polypeptide in which a certain cysteine residue is replaced with serine residue in the amino acid sequence of human interleukin-2 (IL-2) shows the interleukin-2 activity [Science, volume 224, page 1431 (1984)].

A product of the gene which is transcribed and translated from the hyperthermostable protease gene of the present invention is an enzyme precursor (preproenzyme) containing two regions, one of them is a signal peptide necessary for extracellular secretion and the other is a propeptide which is removed upon expression of the activity. When a transformant to which the above gene has been transferred can cleave this signal peptide, an enzyme precursor (proenzyme) from which the signal peptide has been removed is extracellularly secreted. Further, an active form enzyme from which the propeptide has been removed is produced by the self-digestion reaction between proenzymes. All of the preproenzyme, proenzyme and active form enzyme thus obtained from the gene of the present invention are proteins which finally have the equivalent function and fall within the scope of "functional equivalents".

As apparent to those skilled in the art, an appropriate signal peptide may be selected depending upon a host used for the expression of a gene of interest. The signal peptide may be removed when the extracellular secretion is not desired. Therefore, among hyperthermostable protease genes disclosed herein, the genes from which a portion encoding a signal peptide has been removed, and the genes where the portion is replaced with other nucleotide sequence are also within the scope of the present invention in the context that they encode the proteases showing the essentially equivalent activity.

As used herein, a gene which "hybridizes to a hyperthermostable protease gene" refers to a gene which hybridizes with the hyperthermostable protease gene under stringent conditions, that is, those where incubation is carried out at 50 °C for 12 to 20 hours in 6 × SSC (1 × SSC represents 0.15M NaCl, 0.015M sodium citrate, pH7.0) containing 0.5% SDS, 0.1% bovine serum albumin (BSA), 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400 and 0.01% denatured salmon sperm DNA.

BRIEF DESCRIPTION OF DRAWINGS

Fig. 1 is a figure showing a restriction map of a DNA fragment derived from Pyrococcus furiosus contained in the plasmid pTPR12 and the plasmid pUBP13.

Fig. 2 is a figure showing a design of the oligonucleotide PRO-1F.

Fig. 3 is a figure showing a design of the oligonucleotide PRO-2F and PRO-2R.

Fig. 4 is s figure showing a design of the oligonucleotide PRO-4R.

Fig. 5 is a restriction map of the plasmid p2F-4R.

Fig. 6 is a restriction map of the plasmid pTC3.

Fig. 7 is a restriction map of the plasmid pTCS6.

Fig. 8 is a restriction map of the plasmid pTC4.

Fig. 9 is a figure showing the procedures for constructing the plasmid pSTC3.

Fig. 10 is a restriction map of the plasmid pSTC3.

Fig. 11 is a figure comparing the amino acid sequences of the various proteases.

Fig. 12 is a continuation of Fig. 11.

Fig. 13 is a figure showing a restriction map of the Pyrococcus furiosus chromosomal DNA around the protease PFUS gene.

Fig. 14 is a restriction map of the plasmid pSPT1.

Fig. 15 is a restriction map of the plasmid pSNP1.

Fig. 16 is a restriction map of the plasmid pPS1.

Fig. 17 is a restriction map of the plasmid pNAPS1.

Fig. 18 is a figure showing the optimum temperature for the enzyme preparation TC-3.

Fig. 19 is a figure showing the optimum temperature for the enzyme preparation NAPS-1.

Fig. 20 is a figure showing the optimum pH for the enzyme preparation TC-3.

Fig. 21 is a figure showing the optimum pH for the enzyme preparation NP-1.

Fig. 22 is a figure showing the optimum pH for the enzyme preparation NAPS-1.

Fig. 23 is a figure showing the thermostability of the enzyme preparation TC-3.

Fig. 24 is a figure showing the thermostability of the enzyme preparation NP-1.

Fig. 25 is a figure showing the thermostability of the activated enzyme preparation NP-1.

Fig. 26 is a figure showing the thermostability of the enzyme preparation NAPS-1.

Fig. 27 is a figure showing the pH-stability of the enzyme preparation NP-1.

Fig. 28 is a figure showing the stability of the enzyme preparation NP-1 in the presence of SDS.

Fig. 29 is a figure showing the stability of the enzyme preparation NAPS-1 in the presence of SDS.

Fig. 30 is a figure showing the stability of the enzyme preparation NAPS-1 in the presence of acetonitrile.

Fig. 31 is a figure showing the stability of the enzyme preparation NAPS-1 in the presence of urea.

Fig. 32 is a figure showing the stability of the enzyme preparation NAPS-1 in the presence of guanidine hydrochloride.

PREFERRED EMBODIMENTS OF THE INVENTION

The hyperthermostable protease gene of the present invention can be obtained by screening the gene library of hyperthermophiles. As a hyperthermophile, bacteria belonging to the genus Pyrococcus can be used and the gene of interest can be obtained by screening a cosmid library of Pyrococcus furiosus genome.

For example, Pyrococcus furiosus DSM3638 can be used as Pyrococcus furiosus, and the strain is available from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH.

One example of the cosmid libraries of Pyrococcus furiosus genome can be obtained by ligating DNA fragments which are obtained by partial digestion of the genomic DNA of Pyrococcus furiosus DSM3638 with a restriction enzyme Sau3A1 (manufactured by Takara Shuzo Co., Ltd.), with the triple helix cosmid vector (manufactured by Stratagene), and packaging the ligated product into a lambda phage particle according to the in vitro packaging method. Then, the library is transduced into a suitable Escherichia coli, for example, Escherichia coli DH5αMCR (manufactured by BRL) to obtain the transformants, followed by cultivation them, collecting the microbial cells, subjecting them to heat treatment (for example, 100 °C for 10 minutes), sonicating and subjecting them to heat treatment (for example, 100 °C for 10 minutes) again. The presence or absence of the protease activity in the resulting lysate can be screened by utilizing the gelatin-containing SDS-polyacrylamide gel electrophoresis.

In this manner, a cosmid clone containing a hyperthermostable protease gene expressing a protease which is resistant to the above heat treatment can be obtained.

Further, the cosmid DNA prepared from the obtained cosmid clone can be digested into fragments with a suitable restriction enzyme to obtain a recombinant plasmid with an incorporated fragment. Then, a suitable microorganism is transformed with the plasmid, and the protease activity expressed by the resulting transformant can be examined to obtain a recombinant plasmid containing a hyperthermostable protease gene of interest.

That is, the cosmid prepared from one of the above cosmid clones is digested with NotI and PvuII (both manufactured by Takara Shuzo Co., Ltd.) to give an about 7.5kb DNA fragment which can be isolated and inserted between the NotI site and the SmaI site of the plasmid vector pUC19 (manufactured by Takara Shuzo Co., Ltd.) into which the NotI linker (manufactured by Takara Shuzo Co., Ltd.) has been introduced. The plasmid was designated the plasmid pTPR12 and Escherichia coli JM109 transformed with the plasmid was designated Escherichia coli JM109/pTPR12 and has been deposited at National Institute of Bioscience and Human-Technology at 1-1-3. Higashi, Tsukuba-shi, Ibaraki-ken, Japan since May 24, 1994 (original deposit date) as the accession number FERM BP-5103 under Budapest Treaty.

The lysate of the Escherichia coli JM109/pTPR12 shows the similar protease activity to that of the above cosmid clone on the gelatin-containing SDS-polyacrylamide gel.

The nucleotide sequence of the DNA fragment, derived from Pyrococcus furiosus, which was inserted into the plasmid pTPR12 can be determined by a conventional method, for example, the dideoxy method. The nucleotide sequence

of the 4.8kb portion flanked by two DraI sites within the DNA fragment inserted into the plasmid pTPR12 is shown in SEQ ID No. 7 of the Sequence Listing. The amino acid sequence of a gene product deduced from the nucleotide sequence is shown in SEQ ID No. 8 of the Sequence Listing. Thus, a hyperthermostable protease, the nucleotide sequence and the amino acid sequence of which were revealed, derived from Pyrococcus furiosus was designated the protease PFUL. As shown in SEQ ID No. 8 of the Sequence Listing, the protease PFUL is a protease consisting of 1398 residues and having a high-molecular weight of more than 150 thousands.

The protease PFUL gene can be expressed using Bacillus subtilis as a host. As Bacillus subtilis, Bacillus subtilis DB104 can be used and the strain is the known one described in Gene, volume 83, page 215-233 (1989). As a cloning vector, the plasmid pUB18-P43 can be used and the plasmid was gifted from Dr. Sui-Lam Wong at Calgary University. The plasmid contains the kanamycin resistant gene as a selectable marker.

There is the plasmid pUBP13 where an about 4.8kb DNA fragment obtained by digestion of the plasmid pTPR13 with DraI has been inserted into the SmaI site of the plasmid vector pUB18-P43. In the plasmid, the protease PFUL gene is located downstream of the P43 promoter [J. Biol. Chem., volume 259, page 8619-8625 (1984)] which functions in Bacillus subtilis. Bacillus subtilis DB104 transformed with the plasmid was designated Bacillus subtilis DB104/pUBP13. The lysate of the transformant shows the similar protease activity to that of Escherichia coli JM109/pTPR12.

However, only a trace amount of the protease activity is detected in a culture supernatant of the transformant. This is thought to be due to that a molecular weight of the protease PFUL is extremely high and it is not translated effectively in Bacillus subtilis, and that a signal sequence encoded by the protease PFUL gene dose not function well in Bacillus subtilis. There is a possibility that the protease PFUL is a membrane-bound type protease, and the peptide chain on the C-terminal side of the protease PFUL may be a region for binding to the cell membrane.

Fig. 1 shows a restriction map around the protease PFUL gene on the Pyrococcus furiosus chromosome, as well as a DNA fragment inserted into the plasmid pTPR12 and that inserted into the plasmid pUBP13. In addition, an arrow in Fig. 1 shows the open reading frame encoding the protease PFUL.

By comparing the amino acid sequence of the protease PFUL represented by SEQ ID. No. 8 of the Sequence Listing with that of a protease derived from the known microorganism, it is seen that there is homology between the amino acid sequence of the front half portion of the protease PFUL and that of a group of alkaline serine proteases, a representative of which is subtilisin [Protein Engineering, volume 4, page 719-737 (1991)], and that there is an extremely high homology around four amino acid residues which are considered to be important for catalytic activity of the proteases.

As it was revealed that regions commonly present in the proteases derived from a mesophile are conserved in the amino acid sequence of the protease PFUL produced by the hyperthermophile Pyrococcus furiosus, it is expected that these regions are present in the same kind of proteases produced by the hyperthermophiles other than Pyrococcus furiosus.

That is, a DNA having the suitable length can be prepared based on the sequence of a portion encoding the amino acid sequence of a region having the high homology with that of subtilisin and the like, and the DNA can be used as a probe for hybridization or as a primer for gene amplification such as PCR and the like to screen a hyperthermostable protease gene similar to the present enzyme present in various hyperthermophiles.

In the above method, a DNA fragment containing only a portion of the gene of interest is obtained in some cases. Upon this, the nucleotide sequence of the resulting DNA fragment is investigated and confirmed that it is a portion of the gene of interest and, thereafter, hybridisation can be carried out using the DNA fragment or a part thereof as a probe or PCR can be carried out using a primer synthesized based on the nucleotide sequence of the DNA fragment to obtain the whole gene of interest.

The above hybridization can be carried out under the following conditions. That is, a membrane to which a DNA is fixed is incubated with a probe suitably labeled at 50 °C for 12 to 20 hours in 6 × SSC (1 × SSC represents 0.15M NaCl, 0.015M sodium citrate, pH 7.0) containing 0.5% SDS, 0.1% bovine serum albumin (BSA), 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400 and 0.01% denatured salmon sperm DNA. After the completion of incubation, the membrane is washed, beginning with washing at 37 °C in 2 × SSC containing 0.5% SDS, varying the SSC concentration in a range of to 0.1 × and a temperature in a range of to 50 °C, until a signal from a probe hybridized to the fixed DNA can be discriminated from the background.

In addition, it is apparent to those skilled in the art that a probe and a primer can be made based on the thus obtained new hyperthermostable gene to obtain another hyperthermostable protease gene according to the similar method.

Figs. 2, 3 and 4 show the relationship among the amino acid sequences of regions in the amino acid sequence of the protease PFUL which have high homology with those of subtilisin and the like, the nucleotide sequence of the protease PFUL gene encoding the region, and the nucleotide sequences of the oligonucleotides PRO-1F, PRO-2F, PRO-2R and PRO-4R which were synthesized based thereon. Further, SEQ ID Nos. 9, 10, 11 and 12 of the Sequence Listing show the nucleotide sequences of the oligonucleotides PRO-1F, PRO-2F, PRO-2F and PRO-4R. That is, SEQ ID Nos.

9-12 are the nucleotide sequences of one example of the oligonucleotides used for screening the hyperthermostable protease gene of the present invention.

By using a combination of the oligonucleotides as primer, a protease gene can be detected by PCR using a chromosomal DNA of the various hyperthermophiles as a template.

As the hyperthermophiles, the bacteria belonging to the genus Pyrococcus, genus Thermococcus, genus Staphylothermus, genus Thermobacteroides and the like can be used. As the bacteria belonging to genus Thermococcus, for example, Thermococcus celer DSM2476 can be used and the strain can be obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH. When PCR is carried out using a chromosomal DNA of Thermococcus celer DSM2476 as a template and using a combination of the oligonucleotides PRO-1F and PRO-2R or a combination of the oligonucleotides PRO-2F and PRO-4R as a primer, the specific amplification of a DNA fragment is observed and the presence of a protease gene can be identified. In addition, the amino acid sequence encoded by the DNA fragment can be estimated by inserting the DNA fragments into a suitable plasmid vector to make a recombinant plasmid and, thereafter, determining the nucleotide sequence of the inserted DNA fragment by the dideoxy method.

A DNA fragments of about 150 bp amplified using the oligonucleotides PRO-1F and PRO-2R and DNA fragment of about 550bp DNA amplified using the oligonucleotides PRO-2F and PRO-4R are inserted into the HincII site of the plasmid vector pUC18 (manufactured by Takara Shuzo Co., Ltd.). The recombinant plasmids are designated plasmid p1F-2R(2) and plasmid p2F-4R, respectively. SEQ ID No. 13 of the Sequence Listing shows the nucleotide sequence of the inserted DNA fragment in the plasmid p1F-2R(2) and the amino acid sequence deduced therefrom and SEQ ID No. 14 of the Sequence Listing shows the nucleotide sequence of the inserted DNA fragment in the plasmid p2F-4R and the amino acid sequence deduced therefrom. In the SEQ ID No. 13 of the Sequence Listing, the nucleotide sequence from the 1st to the 21st nucleotides and that from the 113rd to he 145th nucleotides and, in the SEQ ID No. 14 of the Sequence Listing, the nucleotide sequence from the 1st to the 32nd nucleotides and that from the 532nd to the 564th nucleoti des are the nucleotide sequence derived from the oligonucleotides used in PCR as primers (each corresponding to the oligonucleotides PRO-1F, PRO-2R, PRO-2F and PRO-4R, respectively). The amino acid sequences having the homology with that of the protease PFUL and the alkaline serine proteases derived from the various microorganisms are present in the amino acid sequences represented by SEQ ID Nos. 13 and 14 of the Sequence Listing, indicating that the above PCR-amplified DNA fragments were amplified with the protease gene as a template.

A restriction map of the plasmid p2F-4R is shown in Fig. 5. In Fig. 5, a thick solid line indicates the DNA fragment inserted into the plasmid vector pUC18.

Then, a hyperthermostable protease gene, for example, a gene of the hyperthermostable protease produced by Thermococcus celer can be obtained by screening the gene library of hyperthermostable bacteria using above oligonucleotides or the amplified DNA fragments obtained by the above PCR as a probe.

One example of the gene libraries of Thermococcus celer, there is a library prepared by partially digesting a chromosomal DNA of Thermococcus celer DSM2476 with the restriction enzyme Sau3AI to obtain a DNA fragment, ligating the fragment with the lambda GEM-11 vector (manufactured by Promega) and packaged it into the lambda phage particle using the in vitro packaging method. Then, the library can be transduced into suitable Escherichia coli, for example, Escherichia coli LE392 (manufactured by Promega) to allow to form the plaques on a plate, and plaque hybridization can be carried out using an amplified DNA fragment obtained by the above PCR as a probe to obtain phage clones containing a gene of interest.

Further, a phage DNA prepared from the phage clones thus obtained can be digested with a suitable restriction enzyme, and southern hybridization can be carried out using the above probe to detect a DNA fragment containing a protease gene.

When the phage DNA prepared from the phage clones obtained by the plaque hybridization is digested with KpnI and BamHI (both manufactured by Takara Shuzo Co., Ltd.), an about 5kb DNA fragment is hybridized to the probe, and the about 5kb DNA fragment can be isolated and inserted between the KpnI site and the BamHI site of the plasmid vector pUC119 (manufactured by Takara Shuzo Co., Ltd.) to obtain a recombinant plasmid. The plasmid was designated plasmid pTC3 and Escherichia coli JM109 transformed with the plasmid was designated Escherichia coli JM109/pTC3. A restriction map of the plasmid pTC3 is shown in Fig. 6. In Fig. 6, a thick solid line designates the DNA fragment inserted into the plasmid vector pUC119.

A DNA fragment which does not contain the protease gene within the DNA fragment inserted into the plasmid pTC3 can be removed according to the following procedures. That is, after the plasmid pTC3 is digested with SacI (manufactured by Takara Shuzo Co., Ltd.), southern hybridization is carried out according to the similar procedures described above and it is found that an about 1.9 kb DNA fragment hybridizes to the probe. Then, the about 1.9 kb DNA fragment can be isolated and inserted into the SacI site of the plasmid vector pUC118 (manufactured by Takara Shuzo Co., Ltd.) to make a recombinant vector. The plasmid was designated plasmid pTCS6 and Escherichia coli JM109 transformed with the plasmid was designated Escherichia coli JM109/pTCS6. A restriction map of the plasmid pTCS6 is shown in Fig. 7. In Fig. 7, a thick solid line designates the DNA fragment inserted into the plasmid vector pUC118. By determining the nucleotide sequence of the DNA fragment inserted into the plasmid pTCS6 by the dideoxy method, it can be con-

firmed that a protease gene is present in the DNA fragment. SEQ ID No. 15 of the Sequence Listing shows the nucleotide sequence of the fragment. By comparing the nucleotide sequence with that of the DNA fragment inserted into the plasmid p1F-2R (2) or that of the plasmid p2F-4R represented by SEQ ID No. 13 or 14 of the Sequence Listing, it is seen that the DNA fragment inserted into the plasmid pTCS6 contains the DNA fragment which is also shared by the plasmid p2F-4R but lacks a 5' region of the protease gene.

Like this, the hyperthermostable protease gene, derived from Thermococcus celer, contained in the plasmid pTCS6 lacks a portion thereof. However, as apparent to those skilled in the art, a DNA fragment covering the full length hyperthermostable protease gene can be obtained by (1) screening the gene library once more, (2) conducting southern hybridization using a chromosomal DNA, or (3) obtaining a DNA fragment of a 5' upstream region by PCR using a cassette and a cassette primer (Takara Shuzo Co., Ltd., Genetic Engineering Products Guidance, 1994-1995 edition, page 250-251).

The present inventors selected the method (3). That is, a chromosomal DNA of the Thermococcus celer is completely digested with a few restriction enzymes, followed by ligation with a cassette (manufactured by Takara Shuzo Co., Ltd.) which corresponds to the restriction enzyme used. PCR is carried out using this ligation product as a template and the primer TCE6R (SEQ ID No. 16 of the Sequence Listing shows the nucleotide sequence of the primer TCE6R) and the cassette primer C1 (manufactured by Takara Shuzo Co., Ltd.) as primers. When the above procedures are carried out using the restriction enzyme HindIII (manufactured by Takara Shuzo Co., Ltd.), an about 1.8 kb DNA fragment is amplified, and a DNA fragment of about 1.5 kb which is obtained by digesting above amplified fragment with HindIII and SacI can be inserted into between the HindIII site and the SacI site of the plasmid vector pUC119 to obtain a recombinant plasmid. The plasmid was designated plasmid pTC4 and Escherichia coli JM109 transformed with the plasmid was designated Escherichia coli JM109/pTC4. A restriction map of the plasmid pTC4 is shown in Fig. 8. In Fig. 8, a thick solid line designates the DNA fragment inserted into the plasmid vector pUC119.

By determining the nucleotide sequence of the DNA fragment inserted into the plasmid pTC4 by the dideoxy method, it can be confirmed that a protease gene is present in the DNA fragment. SEQ ID No. 17 of the Sequence Listing shows the nucleotide sequence of the fragment. By comparing the amino acid sequence deduced from the nucleotide sequence with those of the various proteases, it is found that the DNA fragment inserted into the plasmid pTC4 covers the 5' region of the hyperthermostable protease gene which the plasmid pTCS6 lacks. By combining the nucleotide sequence with that of the DNA fragment inserted into the plasmid pTCS6 represented by SEQ ID No. 15 of the Sequence Listing, the nucleotide sequence of the full length hyperthermostable gene derived from Thermococcus celer can be identified. The nucleotide sequence of the open reading frame present in the obtained nucleotide sequence is shown in SEQ ID No. 2 of the Sequence Listing and the amino acid sequence deduced from the nucleotide sequence is shown in SEQ ID No. 1, respectively. Thus, the hyperthermostable protease derived from Thermococcus celer, with the nucleotide sequence encoding it and the amino acid sequence thereof revealed was designated protease TCES. The full length of the protease TCES gene can be reconstituted by combining the inserted DNA fragment of the plasmid pTC4 and that of the plasmid pTCS6.

It is contemplated that the protease activity expressed by the gene can be confirmed by reconstituting the full length protease TCES gene from two DNA fragments contained in pTC4 and pTCS6, and inserting this downstream of the lac promoter of a plasmid to give an expression plasmid which is introduced into Escherichia coli. However, this method affords no transformants into which the expression vector of interest has been introduced, and it is predicted that the production of a product expressed from the gene is harmful or lethal to Escherichia coli. It is contemplated that, in such a case, for example, a protease is extracellularly secreted using Bacillus subtilis as a host to confirm the activity.

As a host for expressing the protease TCES gene in Bacillus subtilis, the Bacillus subtilis DB104 can be used and, as a cloning vector, the plasmid pUB18-P43 can be used.

However, since the host-vector system for Escherichia coli has the advantages that it contains various kind of vectors and transformation can be carried out simply and highly effectively, as many as possible procedures for constructing an expression vector are desirably, if possible, carried out by using Escherichia coli. That is, in Escherichia coli, an optional nucleotide sequence containing a termination codon is inserted between two protease gene fragments derived from the plasmid pTC4 and the plasmid pTCS6 so that the full length protease TCES gene is not reconstituted, thus, making expression of the gene product impossible and, therefore, the construction of a plasmid can be carried out. Then, this inserted sequence can be removed at the final stage to make the expression plasmid pSTC3 of interest shown in Fig. 10.

The procedures for constructing the plasmid pSTC3 shown in Fig. 9 are explained below.

First, the about 1.8 kb HindIII-SspI fragment inserted into the plasmid pTCS6 is inserted between the HindIII site and the EcoRV site of the plasmid vector pBR322 (manufactured by Takara Shuzo Co., Ltd.) to make the recombinant plasmid pBTC5 and, from this plasmid, the DNA fragment between the HindIII site and the KpnI site derived from a multicloning site of the plasmid vector pUC118 and the BamHI site present on the plasmid vector pBR322 are removed to make the plasmid pBTC5HKB.

Then, based on the nucleotide sequence of the protease TCES gene, the primer TCE12 which can introduce the

EcoRI site and the BamHI site in front of an initiation codon of the protease TCES, and the primer TCE20R which can introduce the ClaI site and a termination codon on the 3' side of only one SacI site present in the nucleotide sequenc are synthesized. SEQ ID Nos. 18 and 19 of the Sequence Listing show the nucleotide sequences of the primer TCE12 and the primer TCE20R, respectively.

An about 0.9 kb DNA fragment which has been amplified by PCR using a chromosomal DNA of Thermococcus celer as a template and using these two primers is digested with EcoRI and ClaI (manufactured by Takara Shuzo Co., Ltd.), and inserted between the EcoRI site and the ClaI site of the plasmid pBTC5HKB to obtain the plasmid pBTC6, which has a mutant gene where the nucleotide sequence of 69 bp including a termination codon is inserted into the SacI site of the protease TCES gene.

A ribosome binding site derived from the Bacillus subtilis P43 promoter [J. Biol. Chem., volume 259, page 8619-8625 (1984)] is introduced between the KpnI site and the BamHI site of the plasmid vector pUC18 to make the plasmid pUC-P43. The nucleotide sequences of the synthetic oligonucleotides BS1 and BS2 are shown in SEQ ID Nos. 20 and 21 of the Sequence Listing, respectively. Then, the plasmid pBTC6 is digested with BamHI and SphI (both manufactured by Takara Shuzo Co., Ltd.) to obtain an about 3 kb DNA fragment containing a mutant gene of the protease TCES, which is inserted between the BamHI site and the SphI site of the plasmid pUC-P43 to construct the plasmid pTC12.

All the above procedures for constructing a plasmid can be carried out using Escherichia coli as a host.

The SacI site present in the plasmid vector pUC18-P43 used for cloning into Bacillus subtilis is previously removed, and an about 3 kb KpnI-SphI DNA fragment obtained from the pTC12 can be inserted into between the KpnI site and the SphI site to make the plasmid pSTC2 using Bacillus subtilis DB104 as a host. The plasmid contains a mutant gene of the protease TCES having the P43 promoter and a ribosome binding site sequence on its 5' side. After the plasmid pSTC2 is digested with SacI, and intramolecular ligation is carried out to obtain a recombinant plasmid, from which the inserted sequence contained in the SacI site of the above mutant gene has been removed. The recombinant plasmid was designated plasmid pSTC3, and Bacillus subtilis DB104 transformed with the plasmid was designated Bacillus subtilis DB104/pSTC3 and has been deposited at National Institute of Bioscience and Human-Technology at 1-1-3, Higashi, Tsukuba-shi, Ibaraki-ken, Japan under accession number FERM BP-5635 since December 1, 1995 (original deposit date) according to Budapest Treaty. The transformant is cultured, and a culture supernatant and an extract from the cells were investigated for the protease activity. As a result, the thermostable protease activity is found in both samples.

Fig. 10 shows a restriction map of the plasmid pSTC3. In Fig. 10, a thick solid line designates the DNA fragment inserted into the plasmid vector pUB18-P43.

When the amino acid sequences of the protease PFUL, the protease TCES and subtilisin are aligned so that the regions having the homology coincide with each other as shown in Figs. 11 and 12, it is seen that the protease PFUL has regions which have no homology with the sequence of the protease TCES and that of subtilisin at the C-terminal side thereof as well as between the regions having the homology. From this, it is contemplated that, besides the protease PFUL, a protease having a smaller molecular weight than that of the protease PFUL, such as the protease TCES or subtilisin may be present in Pyrococcus furiosus. In order to search a gene encoding such a protease, southern hybridization can be carried out using a chromosomal DNA prepared from Pyrococcus furiosus as a target, and using a DNA fragment containing the nucleotide sequence within the protease TCES gene, which encoding the amino acid sequence which is well conserved in three proteases, for example, the about 150 bp DNA fragment inserted into the plasmid p1F-2R (2), as a probe. Although, since the DNA fragment used for a probe has also homology with the protease PFUL gene, the gene fragment is detected as a signal depending upon the hybridization conditions, the position of the signal derived from the gene can be previously estimated on each restriction enzyme used for cutting a chromosomal DNA, from the informations on the nucleotide sequence of the protease PFUL gene and the restriction map. When some enzymes are used, in addition to the position predicted on the protease PFUL gene, an another signal is detected as almost the same level, suggesting the possibility that at least one protease is present in Pyrococcus furiosus in addition to the protease PFUL.

For isolating a gene corresponding to the above new signal, a portion of the gene is first cloned to prevent the failure of isolation of the gene, as in the case of the protease TCES, resulted from the expression of the gene product which is harmful or lethal to Escherichia coli. For example, when a chromosomal DNA of Pyrococcus furiosus is digested with the restriction enzymes SacI and SpeI (both manufactured by Takara Shuzo Co., Ltd.) and the digestion products are used to conduct southern hybridization as described above using a fragment of the protease TCES gene as a probe, it was revealed that a new signal corresponding to about 0.6 kb, derived from the new gene, was observed replacing with a signal corresponding to about 3 kb which was observed in the case of digestion only with SacI. This about 0.6 kb SpeI-SacI fragment encodes an amino acid sequence of at maximum around 200 residues and it can not be contemplated to express a protease having the activity. A Pyrococcus furiosus chromosomal DNA digested with SacI and SpeI is subjected to agarose gel electrophoresis to recover a DNA fragment corresponding to about 0.6 kb from the gel.

Then, the fragment is inserted between the SpeI site and the SacI site of the plasmid vector pBluescript SK(-)

(manufactured by Stratagene) and the resulting recombinant plasmid is used to transform Escherichia coli JM109. From this transformant, a clone with a fragment of interest incorporated can be obtained by colony hybridization using the same probe as that used for the above southern hybridization. Whether a plasmid contained in the resulting clone has the sequence encoding a protease or not can be confirmed by conducting PCR using the primers 1FP1, 1FP2, 2RP1 and 2RP2 (the nucleotide sequences of the primers 1FP1, 1FP2, 2RP1 and 2RP2 are shown in SEQ ID Nos. 22, 23, 24, and 25 of the Sequence Listing) made based on the amino acid sequence common to the above various proteases, or by determining the nucleotide sequence of a DNA fragment inserted into the plasmid prepared from the clone. The plasmid in which the existence of a protease gene is confirmed in this manner was designated the plasmid pSS3. The nucleotide sequence of a DNA fragment inserted in the plasmid, and the amino acid sequence deduced therefrom are shown in SEQ ID No. 26 of the Sequence Listing.

The amino acid sequence deduced from the nucleotide sequence of the DNA fragment inserted into the plasmid pSS3 is shown to have the homology with the sequences of subtilisin, the protease PFUL, the protease TCES and the like. A product of a protease gene different from the protease PFUL, a portion of which was obtained newly from Pyrococcus furiosus, was designated protease PFUS. A region encoding a N-terminal side part of the protease, that is, a region 5' of the SpeI site, and a region encoding a C-terminal side part, that is, a gene fragment 3' of the above SacI site can be obtained by the inverse PCR method. If the restriction enzyme sites in the protease PFUS gene and the vicinity thereof in a chromosome are revealed in advance, the inverse PCR can be carried out using an appropriate restriction enzyme. The restriction enzyme sites can be revealed by cutting a chromosomal DNA of Pyrococcus furiosus with various restriction enzymes, and conducting southern hybridization using a DNA fragment inserted into the plasmid pSS3 as a probe. Consequently, it is shown that the SacI site is located on about 3 kb distant 5' side of the SpeI site and the XbaI site is located on about 5 kb distant 3' side of the SacI site.

A primer used for the inverse PCR can be design to anneal at around an end of the SpeI-SacI fragment contained in the plasmid pSS3. The primers designed to anneal at around the SacI site are designated NPF-1 and NPF-2 and a primer designed to anneal at around the SpeI site is designated NPR-3. The nucleotide sequences thereof are shown in SEQ ID Nos. 27, 28 and 29 of the Sequence Listing, respectively.

A chromosomal DNA of Pyrococcus furiosus is digested with SacI or XbaI (both manufactured by Takara Shuzo Co., Ltd.), respectively, which is allowed to intramolecullarly ligate, and this reaction mixture can be used as a template for the inverse PCR. When a chromosomal DNA is digested with SacI, an about 3 kb fragment is amplified by the inverse PCR, which is inserted into the plasmid vector pT7BlueT (manufactured by Novagen) to obtain a recombinant plasmid which was designated plasmid pS322. On the other hand, in a case of a chromosomal DNA digested with XbaI, an about 9 kb fragment is amplified. The amplified fragment is digested with XbaI to obtain an about 5 kb fragment which is recovered and inserted into the plasmid vector pBluescript SK(-) to obtain a recombinant plasmid, which was designated plasmid pSKX5. By combining the results of southern hybridization performed using the SpeI-SacI fragment contained in the plasmid pSS3 as a probe, and those of analysis on the plasmids pS322 and pSKX5 with the restriction enzymes, a restriction map of the protease PFUS gene and the vicinity thereof in a chromosome can be obtained. The restriction map is shown in Fig. 13.

In addition, by analyzing the nucleotide sequence on a 5' fragment inserted into the plasmid pS322 in a 5' direction starting from the SpeI site, the amino acid sequence of an enzyme protein encoded by the region can be deduced. The resulting nucleotide sequence and the amino acid sequence deduced therefrom are shown in SEQ ID No. 30 of the Sequence Listing. Since the amino acid sequence of this region has the homology with that of a protease such as subtilisin or the like, an initiation codon of the protease PFUS can be presumed based on this homology and, thus, primer NPF-4 which can introduce the BamHI site in front of the initiation codon of the protease PFUS can be designed. On the other hand, the nucleotide sequence determined by analyzing the nucleotide sequence of a 3' fragment of the protease PFUS gene inserted into the plasmid pSKX5 in a 5' direction starting from the XbaI site is shown in SEQ ID No. 31 of the Sequence Listing. Based on the nucleotide sequence, the primer NPR-4 which can insert the SphI site into the vicinity of the XbaI site can be designed. The nucleotide sequences of the primers NPF-4 and NPR-4 are shown in SEQ ID Nos. 32 and 33 of the Sequence Listing, respectively. The full length protease PFUS gene can be amplified by using these two primers and using a chromosomal DNA of Pyrococcus furiosus as a template.

The protease PFUS can be expressed in the Bacillus subtilis system, as in a case of the protease TCES. A plasmid for expressing the protease PFUS can be constructed based on the expression plasmid pSTC3 for the protease TCES. First, a DNA fragment containing the full length protease PFUS gene which can be amplified by the PCR is digested with BamHI and SacI to recover an about 0.8 kb fragment encoding a N-terminal part of the enzyme. And this fragment is replaced with the BamHI-SacI fragment, also encoding a N-terminal part of the protease TCES, of the plasmid pSTC3. The resulting expression plasmid encoding a hybrid protein of the protease TCES and the protease PFUS gene was designated the plasmid pSPT1. Fig. 14 shows a restriction map of the plasmid pSPT1.

Then, the above PCR-amplified DNA fragment is digested with SpeI and SphI to give an about 5.7 kb fragment which is isolated and replaced with the SpeI-SphI fragment encoding a C-terminal part of the protease TCES in the plasmid pSPT1. The expression plasmid thus constructed was designated plasmid pSNP1, and Bacillus subtilis DB104

transformed with the plasmid was designated Bacillus subtilis DB104/pSNP1 and has been deposited at National Institute of Bioscience and Human-Technology (NIBH) at 1-1-3, Higashi, Tsukuba-shi, Ibaraki-ken, Japan since December 1, 1995 (original deposit date) under the accession number FERM BP-5634 under Budapest Treaty. Fig. 15 shows a restriction map of the plasmid pSNP1.

The Bacillus subtilis DB104/pSNP1 is cultured and a culture supernatant and an extract from the cells are examined for the protease activity and it is found that the thermostable protease activity is found in both samples.

The nucleotide sequence of a gene encoding the protease PFUS can be determined by digesting a DNA fragment inserted into the plasmid pSNP1 with a restriction enzyme into the appropriate sized fragments, subcloning the fragments into an appropriate cloning vector, and conducting the dideoxy method using the subcloned fragments as a template. SEQ ID No. 34 of the Sequence Listing shows the nucleotide sequence of open reading frame present in the nucleotide sequence thus obtained. In addition, SEQ ID No. 35 of the Sequence Listing shows the amino acid sequence of the protease PFUS deduced from the nucleotide sequence.

Further, also when Bacillus subtilis DB104 transformed with the plasmid pSPT1, Bacillus subtilis DB104/pSPT1, is cultured, the protease activity is found in both a culture supernatant and an extract from the cells. SEQ ID No. 6 of the Sequence Listing shows the nucleotide sequence of open reading frame encoding a hybrid protein of the protease TCES and the protease PFUS. In addition, SEQ ID No. 5 of the Sequence Listing shows the amino acid sequence of the hybrid protein deduced from the nucleotide sequence.

An amount of an expressed protease of the present invention can be increased by utilizing a gene which is highly expressed in Bacillus subtilis, particularly a secretory protein gene. As such a gene, the genes of α-amylase and the various extracellular proteases can be used. For example, an amount of the expressed protease PFUS can be increased by utilizing the promoter and the signal sequence of subtilisin. That is, by ligating the full length protease PFUS gene to downstream of a region encoding the signal sequence of subtilisin gene so that the translation frames of both genes coincide with each other, the protease PFUS can be expressed as a fusion protein under the control of subtilisin gene promoter.

As the promoter and the signal sequence of subtilisin, those of subtilisin gene, which are inserted into the plasmid pKWZ , described in J . Bacteriol., volume 171, page 2657-2665 (1989) can be used. The nucleotide sequence of the gene is described in the above literature for a 5' upstream region containing the promoter sequence and in J. Bacteriol., volume 158, page 411-418 (1984) for a region encoding subtilisin, respectively. Based on these sequences, the primer SUB4 for introducing the EcoRI site upstream of the promoter sequence of the gene, and the primer BmRI for introducing the BamHI site behind a region encoding the signal sequence of subtilisin are synthesized, respectively. SEQ ID Nos. 36 and 37 of the Sequence Listing show the nucleotide sequences of the primers SUB4 and BmR1, respectively. By using the primers SUB4 and BmR1, an about 0.3 kb DNA fragment containing the region encoding from the promoter to the signal sequence of subtilisin gene can be amplified by PCR using the plasmid pKWZ as a template.

The protease PFUS gene ligated downstream of the DNA fragment can be taken from a chromosomal DNA of Pyrococcus furiosus by the PCR method. As a primer which hybridizes with a 5' part of the gene, the primer NPF-4 can be used. In addition, a primer which hybridizes with a 3' part can be made after the nucleotide sequence downstream of a termination codon of the gene is determined. That is, a portion of the nucleotide sequence of the plasmid pSNPD obtained by subcloning an about 0.6 kb fragment, produced by digestion of the plasmid pSNP1 with BamHI, into the BamHI site of the plasmid vector pUC119 is determined (the nucleotide sequence is SEQ ID No. 38 of the Sequence Listing). Based on the sequence, the primer NPM-1 which hybridizes with a 3' part of the protease PFUS gene and which can introduce the SphI site is synthesized. SEQ ID No. 39 of the Sequence Listing shows the sequence of the primer NPM-1.

On the other hand, when the protease PFUS gene is ligated to the above 0.3 kb DNA fragment by utilizing the BamHI site, only one BamHI site present in the gene becomes a barrier to the procedures. The primers mutRR and mutFR for removing this BamHI site by the PCR-mutagenesis method can be made based on the nucleotide sequence of the protease PFUS gene shown in SEQ ID No. 34 of the Sequence Listing. The nucleotide sequences of the primers mutRR and mutRF are shown in SEQ ID Nos. 40 and 41, respectively. In addition, when the BamHI site is removed by utilizing these primers, glycine present at the position 560 in the amino acid sequence of the protease PFUS shown in SEQ ID No. 35 of the Sequence Listing is substituted with valine due to the nucleotide substitution which is introduced into the site.

By using these primers, the protease PFUS gene to be ligated to the promoter to signal sequence-coding region of subtilisin gene can be obtained. That is, two kinds of PCRs are carried out using a chromosomal DNA of Pyrococcus furiosus as a template and using two kinds of pairs of the primers mutRR and NPF-4, and the primers mutFR and NPM-1. Further, the second PCR is carried out using a hetero duplex formed by mixing the DNA fragments amplified by both PCRs as a template, and using the primers NPF-4 and NPM-1. Thus, the full length of the about 2.4 kb protease PFUS gene containing no BamHI site can be amplified.

An about 2.4 kb DNA fragment obtained by digesting the above PCR-amplified DNA fragment with BamHI and SphI is isolated, and replaced with the BamHI-SphI fragment containing the protease PFUS gene in the plasmid pSNP1. The

expression plasmid thus constructed was designated pPS1 and Bacillus subtilis DB104 transformed with the plasmid was designated Bacillus subtilis DB104/pPS1. When the transformant is cultured, the similar protease activity to that in a case of the use of the plasmid pSNP1 is found in both a culture supernatant and an extract from the cells, and it is confirmed that the substitution of the amino acids dose not affect the enzyme activity. Fig. 16 shows a restriction map of the plasmid pPS1.

An about 0.3 kb DNA fragment containing from the promoter to the signal sequence of the subtilisin is digested with EcoRI and BamHI, and substituted with the EcoRI-BamHI fragment containing the P43 promoter and the ribosome binding site in the plasmid pPS1. The expression plasmid thus constructed was designated pNAPS1 and Bacillus subtilis transformed with the plasmid was designated Bacillus subtilis DB104/pNAPS1. The transformant is cultured, a culture supernatant and an extract from the cells are examined for the protease activity to be found that the protease activity is recognized in both samples. An amount of expressed enzyme is increased as compared with Bacillus subtilis DB104/pSN1. Fig. 17 shows a restriction map of the plasmid pNAPS1.

By a similar method to that in a case of the protease TCES gene and the protease PFUS gene, a protease gene having the homology with these genes can be obtained from hyperthermophiles other than Pyrococcus furiosus and Thermococcus celer. However, in PCR using the above oligonucleotides PRO-1F, PRO-2F, PRO-2R and PRO-4R as a primer and using a chromosomal DNA of Staphylothermus marinus DSM3639 and that of Thermobacteroides proteoliticus DSM5265 as a template, the amplification of a DNA fragment as found in Thermococcus celer was not found.

In addition, it is known that the efficiency of gene amplification by PCR is largely influenced by the efficiency of annealing of a 3' terminal part of a primer and a template DNA. Even when the amplification of a DNA by PCR is not observed, a protease gene can be detected by synthesizing and using the oligonucleotides having the different nucleotide sequence from that used this time but encoding the same amino acid sequence. Alternatively, a protease gene can be also detected by conducting southern hybridization using a chromosomal DNA and using the above oligonucleotides or a portion of other hyperthermostable protease genes as a probe.

An about 1 kb DNA fragment encoding the sequence of residue 323 to residue 650 of the amino acid sequence of the protease PFUL represented by SEQ ID No. 8 of the Sequence Listing is prepared, and this can be used as a probe to conduct genomic southern hybridization using a chromosomal DNA of Staphylothermus marinus DSM3639 and that of Thermobacteroides proteoliticus DSM5265. As a result, when the Staphylothermus marinus chromosomal DNA digested with PstI (manufactured by Takara Shuzo Co., Ltd.) is used, a signal is observed at the position of about 4.8 kb. On the other hand, when the Thermobacteroides proteoliticus chromosomal DNA digested with XbaI is used, a signal is observed at the position of about 3.5 kb.

From this, it is revealed that a sequence having the homology with the protease PFUL, the protease PFUS and the protease TCES gene is present also in the Staphylothermus marinus and Thermobacteroides proteoliticus DNA chromosomes. From the DNA fragment thus detected, a gene encoding a hyperthermostable protease present in Staphylothermus marinus or Thermobacteroides proteoliticus can be isolated and identified by using the same method as that when the gene encoding the protease TCES or the protease PFUS is isolated and identified.

The transformant in which the protease TCES gene, a hyperthermostable protease gene of the present invention, is introduced (Bacillus subtilis DB104/pSTC3) expresses a hyperthermostable protease in a culture by culturing at 37 °C in LB medium containing 10 μg/ml kanamycin. After the completion of cultivation, crude enzyme preparation is obtained by subjecting centrifugation of a culture to collect a supernatant, and salting out with ammonium sulfate and dialysis. Thus, the crude enzyme preparation obtained from Bacillus subtilis DB104/pSTC3 was designated TC-3.

According to the similar procedures, a crude enzyme preparation can be obtained from the transformant Bacillus subtilis DB101/pSNP1 in which the protease PFUS gene is introduced, or from the transformant Bacillus subtilis DB104/pSPT1 in which a gene encoding a hybrid protease of the protease TCES and the protease PFUS. Crude enzyme preparations obtained from Bacillus subtilis DB104/pSNP1 and Bacillus subtilis DB104/pSPT1 were designated NP-1 and PT-1, respectively.

Transformant Bacillus subtilis DB104/pNAPS1 in which the protease PFUS gene, a hyperthermostable protease gene of the present invention, is introduced expresses a hyperthermostable protease in the cells or culture under conventional conditions, for example, by culturing at 37 °C in LB medium containing 10 μg/ml kanamycin. After completion of cultivation, cells and culture supernatant are separated by centrifugation, from either of which a crude enzyme preparation of the protease PFUS can be obtained by the following procedures.

When an enzyme is purified from the cells, the cells are first lysed by the lysozyme treatment, the lysate is heat-treated and centrifuged to recover a supernatant. This supernatant can be fractionated with ammonium sulfate and subjected to hydrophobic chromatography to obtain a purified enzyme. The purified enzyme preparation thus obtained from Bacillus subtilis DB104/pNAPS1 was designated NAPS-1.

On the other hand, the culture supernatant is dialyzed and subjected to anion-exchange chromatography. The eluted active fractions can be collected, heat-treated, fractionated with ammonium sulfate, and subjected to hydrophobic chromatography to obtain a purified enzyme of the protease PFUS. The purified enzyme preparation was designated NAPS-1S.

When the purified products NAPS-1 and NAPS-1S thus obtained are subjected to SDS-polyacrylamide gel electrophoresis, both enzyme preparation show a single band corresponding to a molecular weight of about 45 kDa. These two enzyme preparation are substantially the same enzyme preparation which have been converted into a mature (active-type) enzyme by removing a pro-sequence by heat-treatment during the purification procedures.

The protease preparation produced by the transformants in which a hyperthermostable protease gene obtained by the present invention is introduced, for example, TC-3, NP-1, PT-1, NAPS-1 and NAPS-1S have the following enzymatic and physicochemical properties.

(1) Activity

The enzymes obtained in the present invention hydrolyze gelatin to produce the short-chain polypeptides. In addition, the enzymes hydrolyze casein to produce short-chain polypeptides.

In addition, the enzymes obtained in the present invention hydrolyze succinyl-L-leucyl-L-leucyl-L-valyl-L-tyrosine-4-methylcoumarin-7-amide (Suc-Leu-Leu-Val-Tyr-MCA) to produce a fluorescent material (7-amino-4-methylcoumarin).

Further, the enzymes obtained in the present invention hydrolyze succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-p-nitroanilide (Suc-Ala-Ala-Pro-Phe-p-NA) to produce a yellow material (p-nitroaniline).

(2) Method for measuring enzyme activity

The enzyme activity of the enzyme preparations obtained in the present invention can be measured using a synthetic peptide substrate.

The enzyme activity of the enzyme preparation TC-3 obtained in the present invention can be measured using as a substrate Suc-Leu-Leu-Val-Tyr-MCA (manufactured by Peptide Laboratory). That is, the enzyme preparation to be detected for the enzyme activity is appropriately diluted, to 20 μl of the solution is added 80 μl of a 0.1M sodium phosphate buffer (pH 7.0) containing 62.5 μM Suc-Leu-Leu-Val-Tyr-MCA, followed by incubating at 75 °C for 30 minutes. After the reaction is stopped by the addition of 20 μl of 30% acetic acid, the fluorescent intensity is measured at the excitation wavelength of 355 nm and the fluorescence wavelength of 460 nm to quantitate an amount of the generated 7-amino-4-methylcoumarin, and the resulting value is compared with that obtained when incubating without the addition of the enzyme preparation, to investigate the enzyme activity. The enzyme preparation TC-3 obtained by the present invention had the Suc-Leu-Leu-Val-Tyr-MCA hydrolyzing activity measured at pH 7.0 and 75 °C.

In addition, the enzyme activity of the enzyme preparations NP-1, PT-1, NAPS-1 and NAPS-1S can be photometrically measured using Suc-Ala-Ala-Pro-Phe-p-NA (manufactured by Sigma) as a substrate. That is, an enzyme preparation to be detected for the enzyme activity was appropriately diluted, to 50 μl of the solution was added 50 μl of a 0.1M potassium phosphate buffer (pH 7.0) containing Suc-Ala-Ala-Pro-Phe-p-NA (Suc-Ala-Ala-Pro-Phe-p-NA solution), followed by incubating at 95 °C for 30 minutes. After the reaction was stopped by ice-cooling, the absorbance at 405 nm was measured to quantitate an amount of the generated p-nitroaniline, and the resulting value was compared with that when incubating without the addition of the enzyme preparation, to investigate the enzyme activity. Upon this, a 0.2 mM solution of Suc-Ala-Ala-Pro-Phe-p-NA was used for the enzyme preparations NP-1 and PT-1 and a 1 mM solution was used for the enzyme preparations NAPS-1 and NAPS-1S. The enzyme preparations NP-1, PT-1, NAPS-1 and NAPS-1S obtained by the present invention have the Suc-Ala-Ala-Pro-Phe-p-NA hydrolyzing activity at measured pH 7.0 and 95 °C.

(3) Detection of activity on various substrates

The activity of the enzyme preparations obtained in the present invention on the synthetic peptide substrates is confirmed by a method for measuring the enzyme activity described in the above (2). That is, the enzyme preparation TC-3 obtained in the present invention has the Suc-Leu-Leu-Val-Tyr-MCA hydrlyzing activity, and the enzyme preparations NP-1, PT-1, NAPS-1 and NAPS-1A have the Suc-Ala-Ala-Pro-Phe-p-NA hydrlyzing activity, respectively. In addition, the enzyme preparations NP-1, PT-1, NAPS-1 and NAPS-1S were investigated for the Suc-Leu-Leu-Val-Tyr-MCA hydrolyzing activity by the enzyme activity measuring method described in the above (2) used for the enzyme preparation TC-3, and it was shown that these enzyme preparations had the activity to degrade the substrates. Further, the enzyme preparation TC-3 was investigated for the Suc-Ala-Ala-Pro-Phe-p-NA hydrolyzing activity by the enzyme activity measuring method described in the above (2) used for the enzyme preparations NP-1 and PT-1, and the activity to degrade the substrate was recognized. In addition, the activity of the enzyme preparations obtained in the present invention on gelatin can be detected by confirming the degradation of gelatin by an enzyme on the SDS-polyacrylamide gel. That is, the enzyme preparation to be detected for the enzyme activity was appropriately diluted, to 10 μl of the sample solution was added 2.5 μl of a sample buffer (50 mM Tris-HCl, pH 7.5, 5% SDS, 5% 2-mercaptoethanol, 0.005%

Bromophenol Blue. 50% glycerol), followed by treatment at 100 °C for 5 minutes and electrophoresis using 0.1% SDS-10% polyacrylamide gel containing 0.05% gelatin. After the completion of run, the gel was soaked in a 50 mM potassium phosphate buffer (pH 7.0), and incubated at 95 °C for 3 hours to carry out the enzyme reaction. Then, the gel was stained in 2.5% Coomassie Brilliant Blue R-250, 25% ethanol and 10% acetic acid for 30 minutes, and transferred in 7% acetic acid to remove the excess dye over 3 to 15 hours. The presence of the protease activity was detected by the fact that gelatin is hydrolyzed by a protease into peptides which are diffused out of the gel and, consequently, the relevant portion of the gel was not stained with Coomassie Brilliant Blue. The enzyme preparations TC-3, NP-1, PT-1, NAPS-1 and NAPS-1S obtained by the present invention had the gelatin hydrolyzing activity at 95 °C.

In addition, the enzyme preparations NP-1, NAPS-1 and NSPA-1S derived from the protease PFUS gene are recognized to have the gelatin hydrolyzing activity at the almost same positions on the gel in the above activity measuring method. From this, it is shown that, in these enzyme preparations, the processing from a precursor enzyme into a mature type enzyme occurs in the similar manner.

Further, the hydrolyzing activity on casein can be detected according to the same method as that used for detecting the activity on gelatin except that 0.1% SDS-10% polyacrylamide gel containing 0.05% casein is used. The enzyme preparations TC-3, NP-1, PT-1, NAPS-1 and NAPS-1S obtained by the present invention had the casein hydrolyzing activity at 95 °C.

Alternatively, the casein hydrolyzing activity of the enzyme preparations TC-3, NP-1, NAPS-1 and NAPS-1S obtained by the present invention can be measured by the following method. 100 μl of an appropriately diluted enzyme preparation was added to 100 μl of a 0.1M potassium phosphate buffer (pH 7.0) containing 0.2% casein, incubated at 95 °C for 1 hour, and the reaction was stopped by the addition of 100 μl of 15% trichloroacetic acid. An amount of an acid-soluble short-chain polypeptide contained in the supernatant obtained by centrifugation of this reaction mixture was determined from the absorbance at 280 nm and compared with that when incubating without the addition of an enzyme preparation, to investigate the enzyme activity. The enzyme preparations TC-3, NP-1, NAPS-1 and NAPS-1S obtained by the present invention had the casein hydrolyzing activity at 95 °C.

(4) Optimum temperature

The optimum temperature of the enzyme preparation TC-3 obtained by the present invention was investigated using the enzyme activity measuring method shown in the above (2) except for varying a temperature. As shown in Fig. 18, the enzyme preparation TC-3 showed the activity at a temperature of 37 to 95 °C and the optimum temperature thereof was 70 to 80 °C. That is, Fig. 18 is a figure showing the relationship between the activity of the enzyme preparation TC-3 obtained in the present invention and a temperature, and the ordinate shows the relative activity to the maximum activity (%) and the abscissa shows a temperature.

In addition, the optimum temperature of the enzyme preparation NAPS-1 obtained in the present invention was investigated by using the enzyme activity measuring method shown in the above (2) except for varying a temperature. As shown in Fig. 19, the enzyme preparation NAPS-1 had the activity at a temperature between 40 to 110 °C at the measuring conditions of pH 7.0, and the optimum temperature being 80 to 95 °C. That is, Fig. 19 is a figure showing the relationship between the activity of the enzyme preparation NAPS-1 obtained in the present invention and a temperature, and the ordinate shows the relative activity to the maximum activity (%) and the abscissa shows a temperature.

(5) Optimum pH

The optimum pH of the enzyme preparation TC-3 obtained by the present invention was investigated by the enzyme activity measuring method shown in the above (2). That is, the Suc-Leu-Leu-Val-Tyr-MCA solutions were prepared using the buffers having various pHs, and the enzyme activities obtained by using these solutions were compared. As a buffer, a sodium acetate buffer was used at pH 3 to 6, a sodium phosphate buffer at pH 6 to 8, a sodium borate buffer at pH 8 to 9, and a sodium phosphate-sodium hydroxide buffer at pH 10 to 11. As shown in Fig. 20, the enzyme preparation TC-3 shows the activity at pH 5.5 to 9, and the optimum pH was pH 7 to 8. That is, Fig. 20 is a figure showing the relationship between the activity of the enzyme preparation TC-3 obtained in the present invention and pH, and the ordinate shows the relative activity (%) and the abscissa shows pH.

In addition, the optimum pH of the enzyme preparation NP-1 obtained in the present invention was investigated by the enzyme activity measuring method shown in the above (2). That is, the Suc-Ala-Ala-Pro-Phe-pNA solutions were prepared by using the buffers having various pHs, and the enzyme activities obtained by using these solution were compared. As a buffer, a sodium acetate buffer was used at pH 4 to 6, a potassium phosphate at pH 6 to 8, a sodium borate buffer at pH 5 to 10, and a sodium phosphate-sodium hydroxide buffer at pH 10.5. As shown in Fig. 21, the enzyme preparation NP-1 shows the activity at pH 5 to 10, and the optimum pH was pH 5.5 to 8. That is, Fig. 21 is a figure showing the relationship between the activity of the enzyme preparation NP-1 obtained in the present invention and pH, and

the ordinate shows the relative activity (%) and the abscissa shows pH.

Further, the optimum pH of the enzyme preparation NAPS-1 obtained in the present invention was investigated by the enzyme activity measuring method shown in the above (2). That is, the Suc-Ala-Ala-Pro-Phe-pNA solutions were prepared by using the buffers having various pHs, and the enzyme activities obtained by using these solution were compared. As a buffer, a sodium acetate buffer was used at pH 4 to 6, a potassium phosphate at pH 6 to 8, a sodium borate buffer at pH 8.5 to 10. As shown in Fig. 22, the enzyme preparation NAPS-1 shows the activity at pH 5 to 10, and the optimum pH was pH 6 to 8. That is, Fig. 22 is a figure showing the relationship between the activity of the enzyme preparation NAPS-1 obtained in the present invention and pH, and the ordinate shows the relative activity (%) and the abscissa shows pH.

(6) Thermostability

The thermostability of the enzyme preparation TC-3 obtained by the present invention was investigated. That is, the enzyme preparation was incubated at 80 °C in 20 mM Tris-HCl, pH 7.5 for various periods of time, an appropriate amount thereof was taken to measure the enzyme activity by the method shown in the above (2), and the activity was compared with that when not heat-treated. As shown in Fig. 23, the enzyme preparation TC-3 obtained by the present invention had not less than 90% of the activity even after the heat-treatment for 3 hours and, thus, was stable on the above heat-treatment. That is, Fig. 23 is a figure showing the thermostability of the enzyme preparation TC-3 obtained in the present invention, and the ordinate shows the residual activity (%) after the heat-treatment and the abscissa shows time.

In addition, the thermostability of the enzyme preparation NP-1 obtained in the present invention was investigated. That is, the enzyme preparation was incubated at 95 °C in 20 mM Tris-HCl, pH 7.5 for various periods of time, an appropriate aliquot was taken to determine the enzyme activity by the method shown in the above (2), and the enzyme activity was compared with that when not heat-treated. As shown in Fig. 24, the enzyme preparation NP-1 obtained in the present invention is observed to have the remarkably increased enzyme activity when incubated at 95 °C. This is considered to be because a protease produced as a precursor causes the self-catalytic activation during the heat-treatment. In addition, no decrease in the activity was recognized in the heat-treatment for up to 3 hours. That is, Fig. 24 is a figure showing the thermostability of the enzyme preparation NP-1 obtained in the present invention, and the ordinate shows the residual activity (%) after the heat-treatment and the abscissa shows the time.

In addition, the above enzyme preparation NP-1 activated by the heat-treatment was investigated for the thermostability. That is, the enzyme preparation NP-1 was activated by the heat-treatment at 95 °C for 30 minutes, incubated at 95 °C for various periods of time, and the activity was determined as described above to compare with that when not heat-treated. At the same time, buffers having the various pHs (sodium acetate buffer at pH 5, potassium phosphate buffer at pH 7, sodium borate buffer at pH 9, sodium phosphate-sodium hydroxide buffer at pH 11, 20 mM in every case) were used. As shown in Fig. 25, when the activated enzyme preparation NP-1 obtained in the present invention was treated in a buffer at pH 9, it had not less than 90% of the activity after the heat-treatment for 8 hours and approximately 50% of the activity even after the heat-treatment for 24 hours and, thus, being very stable to the above heat-treatment. That is, Fig. 25 is a figure showing the thermostability of the enzyme preparation NP-1 obtained in the present invention, and the ordinate shows the residual activity (%) after the heat-treatment and the abscissa shows the time.

In addition, the enzyme preparation NAPS-1 obtained by the present invention was investigated for the thermostability. That is, a temperature of the enzyme preparation was maintained at 95 °C in 20 mM Tris-HCl, pH 7.5 for various periods of time, an appropriate aliquot was taken to determine the enzyme activity by the method shown in the above (2) to compare with that when not heat-treated. As shown in Fig. 26, the enzyme preparation NAPS-1 obtained by the present invention had not less than 80% of the activity even after the heat-treatment at 95 °C for 3 hours and, thus, being stable against the above heat-treatment. That is, Fig. 26 is a figure showing the thermostability of the enzyme preparation NAPS obtained in the present invention, and the ordinate shows the residual activity (%) after the heat-treatment and the abscissa shows the time.

(7) pH stability

The pH stability of the enzyme preparation NP-1 obtained by the present invention was investigated according to the following procedures. Each 50 µl of 20 mM buffers at various pHs, which contain the enzyme preparation NP-1 activated by the heat-treatment at 95 °C for 30 minutes, was treated at 95 °C for 60 minutes, and an appropriate aliquot was taken to determine the enzyme activity by the method shown in the above (2) to compare with that when not treated. As a buffer, a sodium acetate buffer was used at pH 4 to 6, a potassium phosphate buffer at pH 6 to 8, a sodium borate buffer at pH 9 to 10, a sodium phosphate-sodium hydroxide buffer at pH 11. As shown in Fig. 27, the enzyme preparation NP-1 obtained by the present invention retained not less than 95% of the activity even after the treatment

at 95 °C for 60 minutes at pH between 5 and 11. That is, Fig. 27 is a figure showing the pH stability of the enzyme obtained by the present invention, and the ordinate shows the residual activity (%) and abscissa shows pH.

(8) Stability to detergent

The stability to detergent of the enzyme preparation NP-1 obtained by the present invention was investigated using SDS as detergent. The enzyme preparation NP-1 was activated by the heat-treatment at 95 °C for 30 minutes. Each 50 µl of a solution containing only the enzyme preparation and a solution further containing SDS to the final concentration of 0.1% or 1% was prepared. These solutions were incubated at 95 °C for various periods of time, an appropriate amount thereof was taken to determine the enzyme activity by the method described in the above (2) to compare with that when not treated. As shown in Fig. 28, the activated enzyme preparation NP-1 obtained by the present invention had not less than 80% of the activity after the heat-treatment at 95 °C for 8 hours and approximately 50% of the activity even the after heat-treatment for 24 hours independently of the presence of SDS and, thus, having the high stability even in the presence of SDS. That is, Fig. 28 is a figure showing the stability to SDS of the enzyme preparation NP-1 obtained by the present invention, and the ordinate shows the residual activity (%) and the abscissa shows time.

In addition, the stability to detergent of the enzyme preparation NAPS-1 obtained by the present invention was investigated using SDS as detergent. Each 50 µl of a solution containing only the enzyme preparation NAPS-1 and a solution further containing SDS to the final concentration of 0.1% or 1% was prepared. These solutions was incubated at 95 °C for various periods of time, an appropriate aliquot was taken to determine the enzyme activity by the method described in the above (2) to compare with that when not treated. As shown in Fig. 29, the enzyme preparation NAPS-1 obtained by the present invention had approximately 80% of the activity after the heat-treatment at 95 °C for 3 hours independently of the presence of SDS. That is, Fig. 29 is a figure showing the stability to SDS of the activated enzyme preparation NAPS-1 obtained by the present invention, and the ordinate shows the residual activity (%) and the abscissa shows the time.

When the above results are compared, it is shown that the enzyme preparation NAPS-1 has remarkably decreased residual activity in comparison with the enzyme preparation NP-1. However, this phenomenon is hardly considered to be based on the difference in the stability to SDS of the enzyme proteins themselves contained in both preparations. It is thought to be the cause for the above phenomenon that NAPS-1 which is the purified enzyme preparation has less contaminant proteins as compared with NP-1 and, thereby, the inactivation easily occurs due to self-digestion.

(9) Stability to organic solvent

The stability to an organic solvent of the enzyme preparation NAPS-1 obtained by the present invention was investigated using acetonitrile. Each 50 µl of enzyme preparation NAPS-1 solutions containing acetonitrile to the final concentration of 25% or 50% was incubated at 95 °C for various periods of time, and an appropriate aliquot was taken to determine the activity by the method described in the above (2) to compare with that when not treated. As shown in Fig. 30, the enzyme preparation NAPS-1 obtained by the present invention had the activity of not less than 80% of that before the treatment, even after the treatment at 95 °C for 1 hour in the presence of 50% acetonitrile. That is, Fig. 30 is a figure showing the stability to acetonitrile of the enzyme preparation NAPS-1 obtained by the present invention.

(10) Stability to denaturing agent

The stability to various denaturing agents of the enzyme preparation NAPS-1 obtained by the present invention was investigated using urea and guanidine hydrochloride. Each 50 µl of the enzyme preparation NAPS-1 solution containing urea to the final concentration of 3.2 M or 6.4 M or guanidine hydrochloride to the final concentration of 1 M, 3.2 M or 6.4 M was prepared. These solutions were incubated at 95 °C for various periods of time, an appropriate aliquot was taken to determine the activity by the method described in the above (2) to compare with that when not treated. As shown Fig. 31, the enzyme preparation NAPS-1 obtained by the present invention shows the resistance to urea and had the activity of not less than 70% of that before the treatment, even after the treatment at 95 °C for 1 hour in the presence of 6.4 M urea. That is, Fig. 31 is a figure showing the stability to urea and Fig. 32 is a figure showing the stability to guanidine hydrochloride, and the ordinate indicates the residual activity and the abscissa indicates the time.

(11) Effects of various reagents

The effects of various reagents on the enzyme preparations TCES and NAPS-1 obtained by the present invention were investigated. That is, the above enzyme preparations were treated at 37 °C for 30 minutes in the presence of the various reagents at the final concentration of 1 mM, and an aliquot thereof was taken to determine the enzyme activity by the method described in the above (2) to compare with that (control) when no reagent was added. The results are

shown in Table 1.

Table 1

Reagent	TCES	NAPS-1
Control	100%	100%
EDTA	103.5%	36.1%
PMSF	8.1%	0.1%
Antipain	19.0%	81.9%
Chymostatin	0%	6.6%
Leupeptin	104.5%	89.3%
Pepstatin	105.2%	100.7%
N-ethylmaleimide	82.6%	102.6%

As shown in Table 1, when treated with PMSF (phenylmethanesulfonyl fluoride) and chymostatin, both enzyme preparations had the remarkably decreased activity. In addition, when treated with antipain, the decrease in the activity was observed in TCES, and when treated with EDTA, in NAPS-1, respectively. In a case of other reagents, the large decrease was not observed in the activity.

(12) Molecular weight

A molecular weight of the enzyme preparation NAPS-1 obtained by the present invention was determined by SDS-PAGE using 0.1% SDS-10% polyacrylamide gel. The enzyme preparation NAPS-1 showed a molecular weight of about 45 kDa on SDS-PAGE. On the other hand, the enzyme preparation NAPS-1S showed the same molecular weight as that of the enzyme preparation NAPS-1.

(13) N-terminal amino acid sequence

The N-terminal amino acid sequence of a mature enzyme, the protease PFUS, was determined using the enzyme preparation NAPS-1 obtained by the present invention. The enzyme preparation NAPS-1 electrophoresed on 0.1% SDS-10% polyacrylamide gel was transferred onto the PVDF membrane, and the N-terminal amino acid sequence of the enzyme on the membrane was determined by the automated Edman degradation using a protein sequencer. The N-terminal amino acid sequence of the mature type protease PFUS thus determined is shown in SEQ ID No. 42 of the Sequence Listing. The sequence coincided with the sequence of amino acids 133 to 144 in the amino acid sequence of the protease PFUS represented by SEQ ID No. 35 of the Sequence Listing, and it was shown that the mature protease PFUS is an enzyme consisting of the polypeptides including behind this part. The amino acid sequence of the mature protease PFUS thus revealed is represented by SEQ ID No. 3 of the Sequence Listing. In addition, as described above, there is no influence on the enzyme activity of the protease PFUS independently of whether 428th amino acid (corresponding to 560th amino acid in the amino acid sequence represented by SEQ ID No. 35 of the Sequence Listing) is glycine or valine. Further, within the nucleotide sequence of the protease PUFS gene represented by SEQ ID No. 34 of the Sequence Listing, that of a region encoding the mature type enzyme is shown in SEQ ID No. 4. The 1283rd base in the sequence may be guanine or thimine.

In a case of in vitro gene amplification by PCR, the misincorporation of a nucleotide may occur during the elongation reaction, leading to the nucleotide substitution in the sequence of the resulting DNA. This frequency largely depends upon the kind of the enzyme used for PCR, the composition of the reaction mixture, the reaction conditions, the nucleotide sequence of a DNA to be amplified and the like. However, when a certain region in a gene is simply amplified as performed usually, the frequency is at best around one nucleotide per 400 nucleotides. In the present invention, PCR was used for isolation of a gene of the protease TCES or the protease PFUS or construction of the expression plasmid therefor. The number of nucleotide substitutions in the nucleotide sequence of the resulting gene is, if any, a few nucleotides. Taking into consideration the fact that the nucleotide substitution on a gene dose not necessarily lead to the amino acid substitution in the expressed protein due to degeneracy of translation codons, the number of the possible amino acid substitutions can be evaluated to be at best 2 to 3 in the whole residues. It cannot be denied that the nucleotide sequence of a gene of the protease TCES and the protease PFUS and the amino acid sequence of the proteases disclosed herein are different from natural ones. However, the object of the present invention

is to disclose a hyperthermostable protease having the high activity at high temperature and a gene encoding the same and, therefore, the protease and the gene are not limited to the same enzyme and the same gene encoding the same as the natural ones. And it is clear to those skilled in the art that even a gene having the possible nucleotide substitution can hybridize to a natural gene under the stringent conditions.

Further, in the specification, a method for obtaining a gene of interest is clearly disclosed such that (1) the library for expression cloning is made from a chromosomal DNA of the hyperthermophiles and the expression of the protease activity is screened, (2) a gene possibly expressing the hyperthermostable protease is isolated by hybridization or PCR based on the homology of amino acid sequences, and the enzyme action of expression products of these genes, that is, the hyperthermostable protease activity is confirmed using an appropriate microorganism. Therefore, it can be easily determined by using the above method whether the gene sequence with the mutation introduced encodes a hyperthermostable protease, after a variety of mutations are introduced into the hyperthermostable protease gene of the present invention using the known mutation introducing method. The kind of the mutation to be introduced is not limited to specified ones as long as the gene sequence obtained as a result of the mutation introduction expresses substantially the same protease activity as that of the hyperthermostable protease of the present invention. However, in order that the expressed protein retains the protease activity, the mutation is desirably introduced into a region other than four regions which are conserved in common in the serine proteases.

A mutation can be randomly introduced into any region of a gene encoding the hyperthermostable protease (random mutagenesis), or alternatively, a desired mutation can be introduced into a specified pre-determined position (site-directed mutagenesis). As a method for randomly introducing a mutation, for example, there is a method for chemically treating a DNA. In this case, a plasmid is prepared such that a region into which a mutation is sought to be introduced is partially single-stranded, and sodium bisulfite is acted on this partially single-stranded region to convert a base cytosine into uracil and, thus, introducing a transition mutation from C:G to T:A. In addition, a method for producing a base substitution during a process where a single-stranded part is repaired to double-strand in the presence of [α-S] dNTP is also known. The details of these methods are described in Proc. Natl. Acad. Sci. USA, volume 79, page 1408-1412 (1982), and Gene, volume 64, page 313-319 (1988).

Random mutation can also be introduced by conducting PCR under the conditions where fidelity of the nucleotide incorporation becomes lower. In particular, the addition of manganese to the reaction system is effective and the details of this method are described in Anal. Biochem., volume 224, page 347-355 (1995). As a method for introducing a site-directed mutation, for example, there is a method using a system where a gene of interest is made single-stranded, a primer designed depending upon a mutation sought to be introduced in this single-stranded part is synthesized, and the primer is annealed to the part, which is introduced into in vivo system where only the strand with a mutation introduced is selectively replicated. The details of this method are described in Methods in Enzymology, volume 154, page 367 (1987). For example, a mutation introducing kit, Mutant K manufactured by Takara Shuzo Co., Ltd. can be used. Site-directed mutagenesis can be conducted also by PCR and the details are described of the method in PCR Technology, page 61-70 (1989), edited by Ehlich and published by Takara Shuzo Co., Ltd. Alternatively, for example, LA-PCR in vitro mutagenesis kit manufactured by Takara Shuzo Co., Ltd. can be used. By using the above method, a mutation of substitution, deletion and insertion can be introduced.

Thus, an enzyme having the similar thermostability and optimum temperature to those of the hyperthermostable protease of the present invention but having a little different, for example, optimum pH can be produced in a host by introducing a mutation using as a base the hyperthermostable protease gene of the present invention. In this case, the base nucleotide sequence of the hyperthermostable protease gene is not necessarily limited to the sequence derived from one hyperthermostable protease.

A hybrid gene can be made by recombinating two or more hyperthermostable protease genes having a sequence homologous to each other, such as those disclosed by the present invention, by exchanging the homologous sequence, and the hybrid enzyme encoded by the gene can be produced in a host. Also in a case of a hybrid gene, whether it is a hyperthermostable protease gene can be determined by testing for the enzyme action of the gene product, that is, the protease activity. For example, by using the above plasmid pSPT1, a hybrid protease of which N-terminal part is derived from the protease PFUS and of which C-terminal part is derived from the protease TCES can be produced in Bacillus subtilis, and this hybrid protease has the protease activity at 95 °C.

The hybrid enzyme is expected to have the properties of two or more base enzymes at the same time. For example, when the protease TCES and the protease PFUS disclosed herein are compared, the protease TCES is superior in respect of the extracellular secretion efficiency and the protease PFUS is superior in respect of the thermostability. Since a signal sequence located at a N-terminal of the proteins has the great influence on extracellular secretion efficiency, if an expression plasmid is constructed so that a protein having, in contrast with pSPT1, a N-terminal part derived from the protease TCES and a C-terminal part derived from the protease PFUS is produced, a hyperthermostable protease having the equal thermostability to that of the protease PFUS can be secreted at the equal secretional efficiency to that of the protease TCES. In addition, since a signal sequence is cut from an enzyme when the enzyme is extracellularly secreted, it has little influence on the nature of the enzyme itself. Therefore, when a hyperthermostable

protease is produced using a mesophile, its signal sequence dose not necessarily need to be derived from hyperthermophiles and a signal sequence derived from a mesophile has no problem as long as a protein of interest is extracellularly secreted at a higher efficiency.

In particular, when a signal sequence of a secretory protein which is highly expressed in a host to be used is employed, a higher secretion is expected.

Upon construction of the above hybrid gene, a recombination dose not necessarily need to be conducted site-directedly. Alternatively, a hybrid gene can be made, for example, by mixing two or more DNAs of a hyperthermostable protease gene, which are raw materials for construction of the hybrid gene, fragmenting these with a DNA degrading enzyme and reconstituting these fragments using a DNA polymerase. The details of this method are described in Proc. Natl. Acad. Sci. USA, volume 91, page 10747-10751 (1994). Also in this case, a sequence of a gene encoding a hyperthermostable gene can be isolated and identified from the resulting hybrid genes by examining the hyperthermostable protease activity of expressed proteins as described above. In addition, it is expected that sequences encoding four regions common to the serine proteases are conserved in the sequences of the genes thus obtained.

Therefore, it is clear to those skilled in the art that the resulting hybrid gene can hybridize to a DNA selected from the oligonucleotides PRO-1F, PRO-2F, PRO-2R and PRO-4R having the nucleotide sequences represented by SEQ ID Nos. 9, 10, 11 and 12 of the Sequence Listing by the appropriate hybridization conditions. In addition, it is also clear that a novel hyperthermostable protease gene obtained by the above mutation introduction can hybridize to a gene having a DNA sequence selected from nucleotide sequences represented by SEQ ID Nos. 9, 10, 11 and 12 of the Sequence Listing, for example, the protease PFUL gene by the appropriate hybridization conditions.

In the specification, we described by focusing on obtaining of a hyperthermostable gene. However, a gene encoding a novel protease having both high thermostability and other properties can be made by constructing a hybrid gene of the hyperthermostable protease gene of the present invention and a protease gene having a sequence homology with the hyperthermostable protease gene of the present invention but having no thermostability, for example, by constructing a hybrid gene with a gene of subtilisin to improve the thermostability of subtilisin, to obtain a gene encoding a protease having the properties originally retained by subtilisin and the higher thermostability.

In the present invention, we used Escherichia coli and Bacillus subtilis as a host into which a gene is introduced in order to detect the protease activity retained by a protein encoded by a gene and produce an enzyme preparation. However, hosts into which a gene is introduced are not limited to specified ones. Any hosts can be used as long as a transforming method is established for the hosts, such as Bacillus brevis, Lactobacillus, yeast, mold fungi, animal cells, plant cells, insect cells and the like. Upon this, it is important that a polypeptide is folded such that an expressed protein becomes an active form and this does not result in the harmful or lethal effect. Among hosts listed above, Bacillus brevis, Lactobacillus and mold fungi which are known to secret their products in a medium can be used as a host for mass production of a protease of interest on an industrial scale, in addition to Bacillus subtilis.

Examples

The following Examples further describe the present invention in detail but are not limit the scope thereof.

Example 1

(1) Preparation of oligonucleotide for detection of hyperthermostable protease gene

By comparing the amino sequence of the protease PFUL represented by SEQ ID No. 8 of the Sequence Listing with those of alkaline serine proteases derived from the known bacterium, the homologous amino acid sequences common to them proved to exist. Among them, three regions were selected and the oligonucleotides were designed, which were used as primers for PCR to detect hyperthermostable protease genes.

Figs. 2, 3 and 4 show the relationship among the amino acid sequences corresponding to the above three regions of the protease PFUL, the nucleotide sequences of the protease PFUL gene encoding the regions, and the nucleotide sequences of the oligonucleotides PRO-1F, PRO-2F, PRO-2R and PRO-4R synthesized based thereon. SEQ ID Nos. 9, 10, 11 and 12 show the nucleotide sequences of the oligonucleotides PRO-1F, PRO-2F, PRO-2R and PRO-4R, respectively.

(2) Preparation of chromosomal DNA of Thermococcus celer

10 ml of a culture of Thermococcus celer DSM2476 obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH was centrifuged to collect the cells which were suspended in 100 µl of 50 mM Tris-HCl, pH 8.0 containing 25% sucrose. To this suspension was added 20 µl of 0.5 M EDTA and 10 µl of 10 mg/ml lysozyme, and was incubated at 20 °C for 1 hour, 800 µl of a SET solution (150 mM NaCl, 1mM EDTA, 20mM Tris-HCl, pH 8.0), 50 µl of 10%

SDS and 10 µl of 20 mg/ml proteinase K were added thereto, and was incubated at 37 °C for 1 hour. The reaction was stopped by extraction with phenol-chloroform and precipitated with ethanol to recover a DNA which was dissolved in 50 µl of a TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) to give a chromosomal DNA solution.

(3) Detection of hyperthermostable protease gene by PCR

A PCR reaction mixture was prepared from the above chromosomal DNA of Thermococcus celer and the oligonucleotides PRO-1F and PRO-2R, or PRO-2F and PRO-4R, and a 35 cycles reaction was carried out, each cycle consisting of 94 °C for 1 minute - 55 °C for 1 minute - 72 °C for 1 minute. When an aliquot of these reaction mixture were subjected to agarose gel electrophoresis, amplification of three DNA fragments in case of the using the oligonucleotides PRO-1F and PRO-2R, and one DNA fragments in case of the using the oligonucleotides PRO-2F and PRO-4R were observed. These amplified fragments were recovered from the agarose gel, and the DNA ends thereof were made blunt using a DNA blunting kit (manufactured by Takara Shuzo Co., Ltd.) and phosphorylated using the T4 polynucleotide kinase (manufactured by Takara Shuzo Co., Ltd.). Then, the plasmid vector pUC19 (manufactured by Takara Shuzo Co., Ltd.) was digested with HincII (manufactured by Takara Shuzo Co., Ltd.), the resulting fragments were dephosphorylated at ends thereof by alkaline phosphatase (manufactured by Takara Shuzo Co. Ltd.), mixed with the above PCR-amplified DNA fragments to allow to ligate, followed by introduction into Escherichia coli JM109. Plasmids were prepared from the resulting transformant, and the plasmids with an appropriate size DNA fragment inserted were selected, followed by sequencing of the inserted fragment by the dideoxy method.

Of these plasmids, the amino acid sequence deduced from the nucleotide sequence of the plasmid p1F-2R(2) containing an about 150 bp DNA fragment amplified using the oligonucleotides PRO-1F and PRO-2R, and that deduced from the nucleotide sequence of the plasmid p2F-4R containing an about 550 bp DNA fragment amplified using oligonucleotides PRO-2F and PRO-4R contained sequences having the homology with the amino acid sequences of the protease PFUL, subtilisin and the like. SEQ ID No. 13 of the Sequence Listing shows the nucleotide sequence of the inserted DNA fragment in the plasmid p1F-2R(2) and the amino acid sequence deduced therefrom and SEQ ID NO. 14 of the Sequence Listing shows the nucleotide sequence of the inserted DNA fragment in the plasmid p2F-4R and the amino acid sequence deduced therefrom. In the nucleotide sequence represented by SEQ ID No. 13 of the Sequence Listing, the sequence of 1st to 21st nucleotides and that of 113rd to 145th nucleotides and, in the nucleotide sequence represented by SEQ ID No. 14 of the Sequence Listing, the sequence of 1st to 32nd nucleotides and that of 532nd to 564th nucleotides are the sequences of the oligonucleotides (corresponding to oligonucleotides PRO-1F, PRO-2R, PRO-2F and PRO-4R, respectively) used as primers for PCR.

Fig. 5 shows a figure of a restriction map of the plasmid p2F-4R.

(4) Screening of protease gene derived from Thermococcus celer

The chromosomal DNA of Thermococcus celer was partially digested with the restriction enzyme Sau3AI (manufactured by Takara Shuzo Co., Ltd.), followed by partial repair of the DNA ends using Klenow Fragment (manufactured by Takara Shuzo Co., Ltd.) in the presence of dATP and dGTP. The DNA fragments were mixed with the lambda GEM-11 XhoI Half-Site Arms Vector (manufactured by Promega) to allow to ligate, which was subjected in vitro packaging using Gigapack Gold (manufactured by Stratagene) to prepare a lambda phage library containing the chromosomal DNA fragments of Thermococcus celer. A part of the library was transformed into Escherichia coli LE392 (manufactured by Promega) to form the plaques on a plate, and the plaques were transferred to Hybond-N+ membrane (manufactured by Amersham). After transference, the membrane was treated with 0.5N NaOH containing 1.5M NaCl, then with 0.5M Tris-HCl, pH 7.5 containing 3M NaCl, washed with 6 × SSC, air dried, and irradiated with ultraviolet rays on the UV transilluminator to fix the phage DNA to the membrane.

On the other hand, the plasmid p2F-4R was digested with PmaCI and StuI (both manufactured by Takara Shuzo Co., Ltd.), which was subjected to 1% agarose gel electrophoresis to recover the separated about 0.5 kb DNA fragment. By using this fragment as a template and using Random Primer DNA Labeling Kit Ver.2 (manufactured by Takara Shuzo Co., Ltd.) and [α-^{32}P]dCTP (manufactured by Amersham), a ^{32}P-labeled DNA probe was prepared.

The membrane with the DNA fixed thereto was treated with a hybridization buffer (6 × SSC containing 0.5% SDS, 0.1% SBA, 0.1% polyvinylpyrrolidone, 0.1% Ficoll 400, 0.01% denatured salmon sperm DNA) at 50 °C for 2 hours, and transferred to the same buffer containing the ^{32}P-labeled DNA probe, followed by hybridization at 50 °C for 15 hours. After the completion of hybridization, the membrane was washed with 2 × SSC containing 0.5% SDS at room temperature, then with 1 × SSC containing 0.5% SDS at 50 °C. The membrane was further rinsed with 1 × SSC, air dried and a X-ray film was exposed thereto at -80 °C for 6 hours to obtain an autoradiogram. About 3,000 phage clones were screened and, as a result, one clone containing a protease gene was obtained. Based on the signal on the autoradiogram, the position of this phage clone was found and the plaque corresponding on the plate used for transfer to the membrane was isolated into 1 ml of a SM buffer (50 mM Tris-HCl, pH 7.5, 1M NaCl, 8 mM MgSO4, 0.01% gelatin) con-

taining 1% chloroform.

(5) Detection of phage DNA fragment containing protease gene derived from Thermococcus celer

Transduced Escherichia coli LE392 using the above phage clone was cultured in the NZCMY medium (manufactured by Bio101) at 37 °C for 15 hours to obtain a culture, from which a supernatant was collected to prepare a phage DNA using QIAGEN-lambda kit (manufactured by QIAGEN). The resulting phage DNAs were digested with BamHI, EcoRI, EcoRV, HincII, KpnI, NcoI, PstI, SacI, SalI, SmaI and SphI (all manufactured by Takara Shuzo Co., Ltd.), respectively, followed by agarose gel electrophoresis. Then, DNAs were transferred from the gel to Hybond-N+ membrane according to the southern transfer method described in Molecular Cloning; A Laboratory Manual, 2nd edition (1986), edited by T. Maniatis, et al., published by Cold Spring Harbor Laboratory.

The resulting membrane was treated in a hybridization buffer at 50 °C for 4 hours, and transferred to the same buffer containing the ^{32}P-labeled DNA probe used in Example 1-(4), followed by hybridization at 50 °C for 18 hours. After the completion of hybridization, the membrane was washed in 1 × SSC containing 0.5% SDS at 50 °C, then rinsed with 1 × SSC and air dried. The membrane was exposed to a X-ray film at -80 °C for 6 hours to obtain an autoradiogram. This autoradiogram indicated that an about 9 kb DNA fragment contained a protease gene in case of the phage DNA digested with KpnI.

Then, the phage DNA containing the above protease gene was digested with KpnI, and further digested successively with BamHI, PstI and SphI, followed by 1% agarose gel electrophoresis. According to the similar procedures to those described above, southern hybridization was conducted and it was indicated that an about 5 kb KpnI-BamHI fragment contained a protease gene.

(6) Cloning of DNA fragment containing protease gene derived from Thermococcus celer

The phage DNA containing the above protease gene was digested with KpnI and BamHI, which was subjected to 1% agarose gel electrophoresis to separate and isolate an about 5 kb DNA fragment from the gel. Then, the plasmid vector pUC119 (manufactured by Takara Shuzo Co., Ltd.) was digested with KpnI and BamHI, which was mixed with the above about 5 kb DNA fragment to allow to ligate, followed by introduction into Escherichia coli JM109. Plasmids were prepared form the resulting transformant, the plasmid containing the about 5 kb DNA fragment was selected and designated the plasmid pTC3.

Fig. 6 shows a restriction map of the plasmid pTC3.

(7) Preparation of plasmid pTCS6 containing protease gene derived from Thermococcus celer

The above plasmid pTC3 was digested with SacI, which was electrophoresed using 1% agarose gel, and southern hybridization was carried out according to the same manner as that described in Example 1-(5) for detecting the phage DNA fragment containing a protease gene. A signal on the resulting autoradiogram indicated that an about 1.9 kb DNA fragment obtained by digesting the plasmid pTC3 with SacI contained a hyperthermostable protease gene.

Then, the plasmid pTC3 was digested with SacI, which was subjected to 1% agarose gel electrophoresis to isolate an about 1.9 kb DNA fragment. Then, the plasmid vector pUC118 (manufactured by Takara Shuzo Co., Ltd.) was digested with SacI, which was dephosphorylated using alkaline phosphatase and mixed with the about 1.9 kb fragment to allow to ligate, followed by introduction into Escherichia coli JM109. Plasmids were prepared from the resulting transformant, and the plasmid containing only one molecule of the about 1.9 kb fragment was selected and designated the plasmid pTCS6.

Fig. 7 shows a restriction map of the plasmid pTCS6.

(8) Determination of nucleotide sequence of DNA fragment derived from Thermococcus celer contained in plasmid pTCS6

In order to determine the nucleotide sequence of the protease gene derived from Thermococcus celer inserted into the plasmid pTCS6, the deletion mutants wherein the DNA fragment portion inserted into the plasmid had been deleted in various length were prepared using the Kilo Sequence Deletion Kit (manufactured by Takara Shuzo Co., Ltd.). Among them, several mutants having suitable length of deletion were selected and the nucleotide sequence of each of the inserted DNA fragment parts was determined by the dideoxy method, and these results were combined to determine the nucleotide sequence of the inserted DNA fragment contained in the plasmid pTCS6. SEQ ID No. 15 of the Sequence Listing shows the resulting nucleotide sequence.

(9) Cloning of 5' upstream region of a protease gene derived from Thermococcus celer by PCR using cassette and cassette primer

A 5' upstream region of the protease gene derived from Thermococcus celer was obtained by using LA PCR in vitro cloning kit (manufactured by Takara Shuzo Co., Ltd.).

Based on the nucleotide sequence of the inserted DNA fragment contained in the plasmid pTCS6 represented by SEQ ID No. 15 of the Sequence Listing, the primer TCE6R for use in cassette PCR was synthesized. SEQ ID No. 16 of the Sequence Listing shows the nucleotide sequence of the primer TCE6R.

Then, a chromosomal DNA of Thermococcus celer was completely digested with HindIII (manufactured by Takara Shuzo Co., Ltd.), and the fragments were ligated to the HindIII cassette (manufactured by Takara Shuzo Co., Ltd.) by the ligation reaction. By using this as a template, a PCR reaction mixture containing the primer TCE6R and the cassette primer C1 (manufactured by Takara Shuzo Co., Ltd.) was prepared, a series of reactions, one cycle of 94 °C for one minute, 30 cycles of 94 °C for 30 seconds - 55 °C for 1 minute - 72 °C for 3 minutes, and one cycle of 72 °C for 10 minutes were carried out. An aliquot of this reaction mixture was subjected to agarose gel electrophoresis and an amplified about 1.8 kb fragment was observed. This amplified fragment was digested with HindIII and SacI, and the about 1.5 kb DNA fragment produced was recovered from the gel after agarose gel electrophoresis. The HindIII-SacI digested plasmid vector pUC119 was mixed with the above about 1.5 kb DNA fragment to allow to ligate, followed by introduction into Escherichia coli JM109. The plasmid harboured by the resulting transformant was examined, the plasmid with only one molecule of the 1.5 kb fragment inserted was selected and designated the plasmid pTC4.

Fig. 8 shows a restriction map of the plasmid pTC4.

(10) Determination of nucleotide sequence of DNA fragment derived from Thermococcus celer contained in plasmid pTC4 and protease TCES gene

In order to determine the nucleotide sequence of a protease gene derived from Thermococcus celer inserted into the plasmid pTC4, the deletion mutants wherein the DNA fragment portion inserted into the plasmid had been deleted in various length were prepared using the Kilo Sequence Deletion Kit. Among them, several mutants having suitable length of deletion were selected and the nucleotide sequence of each of the inserted DNA fragment parts was determined by the dideoxy method, and these results were combined to determine the nucleotide sequence of the inserted DNA fragment contained in the plasmid pTCS4. SEQ ID No. 15 of the Sequence Listing shows the resulting nucleotide sequence.

By combining the sequence with the nucleotide sequence of the inserted DNA fragment contained in the plasmid pTCS6 obtained in Example 1-(8) , the whole nucleotide sequence of the protease gene derived from Thermococcus celer was determined. SEQ ID No. 1 and 2 of the Sequence Listing show the nucleotide sequence of open reading frame present in the nucleotide sequence and the amino acid sequence deduced therefrom of the protease derived from Thermococcus celer, respectively. The protease derived from Thermococcus celer encoded by the gene was designated the protease TCES.

(11) Preparation of plasmid pBTC6 containing protease TCES gene

The plasmid pTCS6 was digested with HindIII and SspI (manufactured by Takara Shuzo Co., Ltd.), which was subjected to 1% agarose gel electrophoresis to recover the separated about 1.8 kb DNA fragment. Then, the plasmid vector pBT322 (manufactured by Takara Shuzo Co., Ltd.) was digested with HindIII and EcoRV, which was mixed with the about 1.8 kb DNA fragment to allow to ligate, followed by introduction into Escherichia coli JM109. Plasmids were prepared from the resulting transformant, the plasmid containing only one molecule of the 1.8 kb fragment was selected and designated the plasmid pBTC5.

Then, the plasmid pBTC5 was completely digested with HindIII and KpnI, which was blunt-ended and was subjected to intramolecular ligation, followed by introduction into Escherichia coli JM109. Plasmids were prepared from the resulting transformant, and the plasmid from which the above two restriction enzyme sites had been removed was selected and designated plasmid pBTC5HK.

Further, the plasmid pBTC5HK was digested with BamHI, which was blunt-ended, and was subjected to intramolecular ligation, followed by introduction into Escherichia coli JM109. Plasmids were prepared from the resulting transformant, the plasmid from which the BamHI site had been removed was selected and designated plasmid pBTC5HKB.

The primer TCE12 which can introduce the EcoRI site and the BamHI site in front of an initiation codon on the protease TCES gene, and the primer TCE20R which has 16 bp-long nucleotide sequence complementary to a 3' part of the SacI site of the plasmid pTCS6 and can introduce the ClaI site and a termination codon were synthesized. SEQ ID Nos. 18 and 19 of the Sequence Listing show the nucleotide sequences of the primer TCE12 and the primer TCE20R, respectively. A PCR reaction mixture was prepared using these two primers and using a chromosomal DNA of Thermo-

coccus celer as a template. A reaction of 25 cycles, each cycle consisting of 94 °C for 30 seconds - 55 °C for 1 minute - 72 °C for 1 minute, was carried out to amplify an about 0.9 kb DNA fragment having these two oligonucleotides on both ends and containing a part of the protease TCES gene.

The above about 0.9 kb DNA fragment was digested with EcoRI and ClaI (manufactured by Takara Shuzo Co., Ltd.), which was mixed with the EcoRI-ClaI digested plasmid pBTC5HKB to allow to ligate, followed by introduction into Escherichia coli JM109. Plasmids were prepared from the resulting transformant, and the plasmid containing only one molecule of the about 0.9 kb fragment was selected and designated plasmid pBTC6.

(12) Preparation of plasmid pTC12 containing protease TCES gene

The plasmid pBTC6 was digested with BamHI and SphI, which was subjected to 1% agarose gel electrophoresis to recover the separated about 3 kb DNA fragment. Then, the plasmid pUC-P43SD where the ribosome binding site sequence derived from Bacillus subtilis P43 promoter was introduced between the KpnI site and the BamHI site of the plasmid vector pUC18 (manufactured by Takara Shuzo Co., Ltd.) (the nucleotide sequence of the synthetic oligonucleotides BS1 and BS2 used for introduction of the sequence are shown in SEQ ID Nos. 20 and 21 of the Sequence Listing) was digested with BamHI and SphI, which was mixed with the previously recovered about 3 kb DNA fragment to allow to ligate, followed by introduction into Escherichia coli JM109. Plasmids were prepared from the resulting transformant, the plasmid containing only one molecule of the above about 3 kb DNA fragment was selected and designated plasmid pTC12.

(13) Preparation of plasmid pSTC3 containing protease TCES gene for transforming Bacillus subtilis

The above plasmid pTC12 was digested with KpnI and SphI, which was subjected to 1% agarose electrophoresis to recover the separated about 3 kb DNA fragment. Then, the plasmid vector pUB18-P43 was digested with SacI, which was bunt-ended and allowed to self-ligate to give the plasmid vector pUB18-P43S from which the SacI site had been removed. This was digested with KpnI and SphI, which was mixed with the previously recovered about 3kb DNA fragment and allowed to ligate, followed by introduction into Bacillus subtilis DB104. Plasmids were prepared from the resulting kanamycin-resistant transformant, and the plasmid containing only one molecule of the above about 3 kb DNA fragment was selected and designated plasmid pSTC2.

Then, the plasmid pSTC2 was digested with SacI and was subjected to intramolecular ligation, followed by introduction into Bacillus subtilis DB104. Plasmids were prepared from the resulting kanamycin-resistant transformant, the plasmid containing only one SacI site and designated plasmid pSTC3.

Then, Bacillus subtilis DB104 harbouring the plasmid pSTC3 was designated Bacillus subtilis DB104/pSTC3.

Fig. 10 shows a restriction map of the plasmid pSTC3.

Example 2

(1) Preparation of chromosomal DNA of Pyrococcus furiosus

Pyrococcus furiosus DMS3638 was cultured as follows. A medium having the composition of 1% trypton, 0.5% yeast extract, 1% soluble starch, 3.5% Jamarin S • Solid (manufactured by Jamarin Laboratory), 0.5% Jamarin S • Liquid (manufactured by Jamarin Laboratory), 0.003% $MgSO_4$, 0.001% NaCl, 0.0001% $FeSO_4 \cdot 7H_2O$, 0.0001% $CoSO_4$, 0.0001% $CaCl_2 \cdot 7H_2O$, 0.0001% $ZnSO_4$, 0.1 ppm $CuSO_4 \cdot 5H_2O$, 0.1 ppm H_3BO_3, 0.1 ppm $KAl(SO_4)_2$, 0.1 ppm $Na_2MoO_4 \cdot 2H_2O$, 0.25 ppm $NiCl_2 \cdot H_2O$ was placed in a 2 liter medium bottle, and was sterilized at 120 °C for 20 minutes, nitrogen gas was blown into the medium to purge out the dissolved oxygen, and the above bacterial strain was inoculated into the medium, followed by subjecting to stationarily culture at 95 °C for 16 hours. After the completion of cultivation, the cells were collected by centrifugation.

Then, the resulting cells were suspended in 4 ml of 50 mM Tris-HCl (pH 8.0) containing 25% sucrose, to this suspension was added 2 ml of 0.2 M EDTA and 0.8 ml of lysozyme (5 mg/ml) and incubated at 20 °C for 1 hour, 24 ml of a SET solution (150 mM NaCl, 1 mM EDTA, 20mM Tris-HCl, pH 8.0), 4 ml of 5% SDS and 400 μl of proteinase K (10 mg/ml) were added thereto and incubated at 37 °C for another 1 hour. The reaction was stopped by extraction with phenol-chloroform, followed by ethanol precipitation to obtain about 3.2 mg of the chromosomal DNA.

(2) Genomic southern hybridization of Pyrococcus furiosus chromosomal DNA

A chromosomal DNA of Pyrococcus furiosus was digested with SacI, NotI, XbaI, EcoRI and XhoI (all manufactured by Takara Shuzo Co., Ltd.), respectively. An aliquot of the reaction mixture was further digested with SacI and EcoRI, which was subjected to 1% agarose gel electrophoresis, followed by southern hybridization according to the procedures

described in Example 1-(5). A ^{32}P-labeled DNA, which was prepared using an about 0.3 kb DNA fragment obtained by digesting the above plasmid p1F-2R(2) with EcoRI and PstI as a template and using BcaBEST DNA Labeling kit (manufacture by Takara Shuzo Co., Ltd.) and [α-^{32}P]dCTP, was used as a probe. A membrane was washed in 2 × SSC containing SDS to the final concentration of 0.5% at room temperature, rinsed with 2 × SSC and the autoradiogram was obtained. As a result, a signal was observed in two DNA fragments of about 5.4 kb and about 3.0 kb produced by digesting a Pyrococcus furiosus chromosomal DNA with SacI and it was indicated that a protease gene was present on respective fragments. When the SacI-digested fragment was further digested with SpeI (manufactured by Takara Shuzo Co., Ltd.), the signal of the above about 5.4 kb fragment did not show the change but the signal which had been seen in the about 3.0 kb fragment was lost, and a signal was newly observed in the about 0.6 kb fragment. Since the SpeI site is not present in the protease PFUL gene represented by SEQ ID No. 7 of the Sequence Listing, it was suggested that a signal on the about 0.6 kb fragment obtained by the digestion with SacI and SpeI was derived from a novel hyperthermostable protease (hereinafter referred to as "protease PFUS"). In addition, regarding the products from digestion of Pyrococcus furiosus chromosomal DNA with XbaI, a signal was observed on two DNA fragments of about 3.3 kb and about 9.0 kb. From a restriction map of protease PFUL gene shown in Fig. 1, it was presumed that the about 3.3 kb fragment contained the protease PFUL gene and the about 9.0 kb fragment contained the protease PFUS gene. When the above chromosomal DNA was digested with XbaI and SacI, a signal was observed on the about 2.0 kb fragment and the about 3.0 kb fragment. From the positions of the SacI and XbaI cleavage sites present on the protease PFUL gene shown in SEQ ID No. 7 of the Sequence Listing, it was presumed that the protease PFUL gene is present on the about 2.0 kb SacI-XbaI fragment. On the other hand, it was presumed that the protease PFUS gene was present on the about 3.0 kb fragment. Combining with the results on the digestion with SacI, it was shown that no XbaI site is present on the about 3.0 kb DNA fragment obtained by the digestion with SacI alone.

(3) Cloning of 0.6 kb SpeI-SacI fragment containing protease PFUS gene

A chromosomal DNA of Pyrococcus furiosus was digested with SacI and SpeI, which was subjected to 1% agarose gel electrophoresis to recover the DNA fragment corresponding to about 0.6 kb from the gel. Then, the plasmid pBluescript SK(-) (manufactured by Stratagene) was digested with SacI and SpeI, which was mixed with the about 0.6 kb DNA fragment to allow to ligate, followed by introduction into Escherichia coli JM109 to obtain the plasmid library containing the chromosomal DNA fragments. Transformed Escherichia coli JM109 was seeded on a plate to form the colonies, and the produced colonies were transferred to a Hybond-N+ membrane, which was incubated at 37 °C for about 2 hours on a new LB plate. This membrane was treated with 0.5N NaOH containing 1.5M NaCl, then with 0.5M Tris-HCl (pH 7.5) containing 1.5 M NaCl, washed with 2 × SSC, air dried and the plasmid DNA was fixed to the membrane by irradiating with ultraviolet rays on a UV transilluminator. This membrane was treated at 50 °C for 2 hours in a hybridization buffer, and transferred to the same buffer containing a ^{32}P-labeled DNA probe used for southern hybridization described in Example 2-(2), to hybridize at 50 °C for 18 hours. After the completion of hybridization, the membrane was washed in 2 × SSC containing 0.5% SDS at room temperature, and washed at 37 °C. Further, the membrane was rinsed with 2 × SSC, air dried, exposed to a X-ray film at -80 °C for 12 hours to obtain an autoradiogram. About 500 clones were screened and, as a result, 3 clones containing a protease gene were obtained. From a signal on the autoradiogram, the positions of these clones were examined and the corresponding colonies on the plate used for transfer to the membrane were isolated in LB medium.

(4) Detection of protease PFUS gene by PCR

Oligonucleotides which used for detection of a hyperthermostable protease gene by PCR as a probe were designed based on the nucleotide sequences encoding two regions having the high homology with the amino acid sequences of alkaline serine proteases derived from the known microorganisms in the protease PFUL gene. Based on the amino acid sequence of the protease PFUL represented by Figs. 2 and 3, the primers 1FP1, 1FP2, 2RP1 and 2RP2 were synthesized. SEQ ID Nos. 22, 23, 24 and 25 of the Sequence Listing show the nucleotide sequences of the oligonucleotides 1FP1, 1FP2, 2RP1 and 2RP2.

PCR reaction mixtures containing the plasmids prepared from the above three clones as well as the oligonucleotides 1FP1 and 2RP1, or 1FP1 and 2RP2, or 1FP2 and 2RP1, or 1FP2 and 2RP2 were prepared, and a 30 cycle reaction was carried out, each cycle consisting of 94 °C for 30 seconds - 37 °C for 2 minutes -72 °C for 1 minute. It was shown that, when aliquots of these reaction mixtures were subjected to agarose gel electrophoresis, respectively, the amplification of an about 150 bp DNA fragment was observed in all the three above plasmids when used the primers 1FP2 and 2RP2, indicating that a protease gene was present on these plasmids.

One of the above three clones was selected and designated plasmid pSS3.

(5) Determination of nucleotide sequence of protease PFUS gene contained in plasmid pSS3

The nucleotide sequence of the inserted DNA fragment in the plasmid was determined by the dideoxy method using the plasmid pSS3 as a template and using the primer M4 and the primer RV (both manufactured by Takara Shuzo Co., Ltd.). SEQ ID No. 26 of the Sequence Listing shows the resultant nucleotide sequence and the amino acid sequence which was deduced to be encoded by the nucleotide sequence. By comparing the amino acid sequence with that of the protease PFUL, the protease TCES and subtilisin, it was presumed that the DNA fragment inserted in the plasmid pSS3 encoded the amino acid sequence having the homology with these proteases.

(6) Cloning of N-terminal coding region and C-terminal coding region of protease PFUS by inverse PCR method

In order to obtain genes encoding N-terminal amino acid sequence and C-terminal one of the protease PFUS, the inverse PCR was carried out. A primer used for the inverse PCR was synthesized based on the nucleotide sequence of the inserted DNA fragment in the plasmid pSS3. SEQ ID Nos. 27, 28 and 29 of the Sequence Listing show the nucleotide sequences of the primers NPF-1, NPF-2 and NPR-3.

A chromosomal DNA of Pyrococcus furiosus was digested with SacI and XbaI and was subjected to intramolecular ligation. PCR mixtures containing an aliquot of the ligation reaction mixture and the primers NPF-1 and NPR-3, or NPF-2 and NPR-3 were prepared and a 30 cycle reaction was carried out, each cycle consisting of 94 °C for 30 seconds - 67 °C for 10 minutes. When an aliquot of this reaction mixture was subjected to agarose gel electrophoresis, an about 3 kb amplified fragment was observed in a case of the use of the primers NPF-2 and NPR-3. This amplified fragment was recovered from the agarose gel, and mixed with the plasmid vector pT7BlueT (manufactured by Novagen) to allow to ligate, followed by introduction into Escherichia coli JM109. Plasmids were prepared from the resultant transformant, the plasmid containing an about 3 kb fragment was selected and designated plasmid pS322.

On the other hand, an about 9 kb amplified fragment was observed in a case of the use of the primers NPF-1 and NPR-3. This amplified fragment was recovered from the agarose gel, the DNA ends were made blunt using a DNA blunting kit, followed by further digestion with XbaI. This was mixed with the plasmid vector pBluescript SK(-) digested with XbaI and HincII to allow to ligate, followed by introduction into Escherichia coli JM109. Plasmids were prepared from the resulting transformant, the plasmid containing an about 5 kb DNA fragment was selected and designated the plasmid pSKX5.

(7) Sequencing of nucleotide sequence of protease PFUS gene contained in plasmid PS322 and pSKX5

The nucleotide sequence of a gene encoding a N-terminal region of the protease PFUS was determined by the dideoxy method using the plasmid pS322 as a template and using the primer NPR-3. SEQ ID No. 30 of the Sequence Listing shows a part of the resulting nucleotide sequence and the amino acid sequence deduced to be encoded by the nucleotide sequence.

Further, the nucleotide sequence of a region corresponding to a 3' part of the protease PFUS gene was determined by the dideoxy method using the plasmid pSKX5 as a template and using the primer RV. SEQ ID No. 31 of the Sequence Listing shows a part of the resulting nucleotide sequence.

(8) Synthesis of primer used for amplification of full length protease PFUS gene

Based on the nucleotide sequence obtained in Example 2-(7), a primer used for amplification of the full length of the protease PFUS gene was designed. Based on the nucleotide sequence encoding a N-terminal part of the protease PFUS shown in SEQ ID No. 30 of the Sequence Listing, the primer NPF-4 which can introduce BamHI site in front of an initiation codon of the protease PFUS gene. SEQ ID No. 32 of the Sequence Listing shows the nucleotide sequence of the primer NPF-4. In addition, based on the nucleotide sequence in the vicinity of a 3' region of the protease PFUS shown in SEQ ID No. 31 of the Sequence Listing, the primer NPR-4 having a sequence complementary to the nucleotide sequence and a SphI site was synthesized. SEQ ID No. 33 of the Sequence Listing shows the nucleotide sequence of the primer NPR-4.

(9) Preparation of plasmid pSPT1 containing hybrid gene of protease derived from Pyrococcus furiosus and protease TCES, for transformation of Bacillus subtilis

By using a LA PCR kit (manufactured by Takara Shuzo Co., Ltd.), a PCR reaction mixture (hereinafter a PCR reaction mixture prepared by using a LA PCR kit is referred to as "LA-PCR reaction mixture") containing the primers NPF-4 and NPR-4 and a chromosomal DNA of Pyrococcus furiosus, and a reaction of 30 cycles, each cycle consisting of 94 °C for 20 seconds - 55 °C for 1 minute - 68 °C for 7 minutes, was carried out to amplify an about 6 kb DNA fragment

having these two primers on both ends and containing the coding region of the protease PFUS gene.

The about 6 kb DNA fragment was digested with BamHI and SacI, which was subjected to 1% agarose gel electrophoresis to recover the separated about 0.8 kb DNA fragment. This fragment was mixed with the plasmid pSTC3 digested with BamHI and SacI to allow to ligate, followed by introduction into Bacillus subtilis DB104. Plasmids were prepared from the resultant kanamycin-resistant transformant, and the plasmid containing only one molecule of the above 0.8 kb fragment was selected and designated the plasmid pSPT1.

Bacillus subtilis DB104 harboring the plasmid pSPT1 was designated Bacillus subtilis DB104/pSTP1.

Fig. 14 shows a restriction map of the plasmid pSPT1.

(10) Preparation of plasmid pSNP1 containing protease PFUS gene for transformation of Bacillus subtilis

The about 6 kb DNA fragment amplified in Example 2-(9) was digested with SpeI and SphI, which was subjected to 1% agarose gel electrophoresis to recover the separated about 5.7 kb DNA fragment. This was mixed with the plasmid digested with SpeI and SphI to allow to ligate, followed by introduction into Bacillus subtilis DB104. Plasmids were prepared from the resulting kanamycin-resistant transformant, and the plasmid containing only one molecule of the 5.7 kb fragment was selected and designated the plasmid pSNP1. Bacillus subtilis transformed with the plasmid pSNP1 was designated as Bacillus subtilis DB104/pSNP1.

Fig. 15 shows a restriction map of the plasmid pSNP1.

(11) Determination of nucleotide sequence of protease PFUS gene contained in plasmid pSNP1

An about 6 kb DNA fragment containing a protease gene inserted into the plasmid pSNP1 was fragmented into appropriate size with a variety of restriction enzymes, and the fragments were subcloned into the plasmid vector pUC119 or pBluescript SK(-). The nucleotide sequence was determined by the dideoxy method using the resulting recombinant plasmid as a template and using a commercially available universal primer. Regarding a part from which the fragments having appropriate size could not be obtained, the primer walking method was used utilizing the synthetic primers. The nucleotide sequence of an open reading frame present in the nucleotide sequence of the DNA fragment inserted into the plasmid pSNP1 thus determined, and the amino acid sequence of a protease derived from Pyrococcus furiosus deduced from the nucleotide sequence are shown in SEQ ID Nos. 34 and 35, respectively.

(12) Synthesis of primer for amplification of protease PFUS gene

In order to design a primer, which is used for amplification of the full length protease PFUS gene and hybridizes to a 3' part of the gene, the nucleotide sequence of the 3' part of the gene was determined. First, an about 0.6 kb DNA fragment containing the 3' region of the protease PFUS gene, obtained by digestion of the plasmid pSNP1 with BamHI, was ligated with the plasmid vector pUC119 which had been digested with BamHI and dephosphorylated with alkaline phosphatase. The resulting recombinant plasmid was designated plasmid pSNPD and the nucleotide sequence of a region corresponding to the 3' part of the protease PFUS gene was determined by the dideoxy method using this as a template. SEQ ID No. 38 of the Sequence Listing shows the nucleotide sequence, from the BamHI site to 80 bp upstream nucleotide, present in the region (the sequence of the complementary chain). Then, based on the sequence, the primer NPM-1 which hybridizes to a 3' part of the protease PFUS gene and contains a SphI site was synthesized. SEQ ID No. 39 of the Sequence Listing shows the nucleotide sequence of the primer NPM-1.

In addition, the primers mutRR and mutFR for elimination the BamHI sites which are present about 1.7 kb downstream from an initiation codon within the protease PFUS gene were synthesized. SEQ ID Nos. 40 and 41 of the Sequence Listing show the nucleotide sequences of the primers mutRR and mutFR, respectively.

(13) Preparation of plasmid pPS1 containing full length protease PUFS gene

Two sets of LA-PCR reaction mixtures containing Pyrococcus furiosus chromosomal DNA as a template and a combination of the primers NPF-4 and mutRR or a combination of the primers mutFR and NPM-1 were prepared, and a reaction of 30 cycles, each cycle consisting of 94 °C for 30 seconds - 55 °C for 1 minute - 68 °C for 3 minutes, was carried out. When agarose gel electrophoresis was carried out using an aliquot of this reaction mixture, an about 1.8 kb DNA fragment was amplified in a case of the use of the primer NPF-4 and mutRR, and an about 0.6 kb DNA fragment in a case of the use of the primers mutFR and NMP-1.

Each amplified DNA fragment from which the primers had been removed by using SUPREC-02 (manufactured by Takara Shuzo Co., Ltd.) was prepared from the two set of the PCR mixture. A LA-PCR reaction mixture containing both of these amplified DNA fragments and not containing the primers and LA Taq was prepared, which was used to carry out heat denaturation at 94 °C for 10 minutes, followed by cooling to 30 °C over 30 minutes and maintaining at 30 °C

for 15 minutes to form a hetero duplex. Then, to this reaction mixture, LA Taq was added and was incubated at 72 °C for 3 minutes, the primers NPF-4 and NPM-1 were added thereto and a reaction of 25 cycles, each cycle consisting of 94 °C for 30 seconds - 55 °C for 1 minute - 68 °C for 3 minutes, was carried out. Amplification of an about 2.4 kb DNA fragment was observed in this reaction mixture.

The about 2.4 kb DNA fragment was digested with BamHI and SphI, the fragments were mixed with the plasmid pSNP1, described in Example 2 -(11), from which the full length protease PFUS gene had been removed previously by digestion with BamHI and SphI, to allow to ligate, followed by introduction into Bacillus subtilis DB104. Plasmids were prepared from the resulting kanamycin-resistant transformant, and the plasmid with only one molecule of the about 2.4 kb fragment inserted was selected and designated plasmid pPS1. Bacillus subtilis DB104 transformed with the plasmid DB104 was designated Bacillus subtilis DB104/pPS1.

Fig. 16 shows a restriction map of the plasmid pPS1.

(14) Amplification of DNA fragment of a region from the promoter to the signal sequence of subtilisin gene

A primer for obtaining a region from promoter to signal sequence of subtilisin gene was synthesized. First, with reference to the nucleotide sequence of a promoter region of subtilisin gene described in J. Bacteriol., volume 171, page 2657-2665 (1989), the primer SUB4 which hybridizes to a part upstream of the region and contains the EcoRI site was synthesized (SEQ ID No. 36 of the Sequence Listing shows the nucleotide sequence of the primer SUB4). Then, with reference to the nucleotide sequence of a region encoding subtilisin described in J. Bacteriol., volume 158, page 411-418 (1984), the primer BmR1 which can be introduce the BamHI site just behind the signal sequence was synthesized (SEQ ID No. 37 of the Sequence Listing shows the nucleotide sequence of the primer BmR1).

The plasmid pKWZ containing subtilisin gene described in J. Bacteriol., volume 17, page 2657-2665 (1989) was used as a template to prepare a PCR reaction mixture containing the primers SUB4 and BmR1, and a reaction of 30 cycles, each cycle consisting of 94 °C for 30 seconds - 55 °C for 1 minute - 68 °C for 2 minutes, was carried out. Agarose gel electrophoresis of an aliquot of this reaction mixture confirmed amplification of an about 0.3 kb DNA fragment.

(15) Preparation of plasmid pNAPS1 containing protease PFUS gene for transformation of Bacillus subtilis

The about 0.3 kb DNA fragment was digested with EcoRI and BamHI, which was mixed with the plasmid pPS1, described in Example 2-(13), which previously had been digested with EcoRI and BamHI to allow to ligate, followed by introduction into Bacillus subtilis DB104. Plasmids were prepared from the resulting kanamycin-resistant transformant and the plasmid containing only one molecule of the about 0.3 kb fragment was selected and designated the plasmid pNAPS1. In addition, Bacillus subtilis DB104 transformed with the plasmid pNAPS1 was designated Bacillus subtilis DB104/pNAPS1.

Fig. 17 shows a restriction map of the plasmid pNAPS1.

Example 3

(1) Preparation of probe for detecting hyperthermostable protease gene

The plasmid pTPR12 containing the protease PFUL gene was digested with BalI and HincII (both manufactured by Takara Shuzo Co., Ltd.), which was subjected to 1% agarose gel electrophoresis to recover the separated about 1 kb DNA fragment. A ^{32}P-labeled DNA probe was prepared using the DNA fragment as a template and using BcaBEST DNA labeling kit and [α-^{32}P] dCTP.

(2) Detection of hyperthermostable protease gene present in hyperthermophile Staphylothermus marinus and Thermobacteroides proteoliticus

Chromosomal DNAs were prepared from each 10 ml of cultures of Staphylothermus marinus DSM3639 and Thermobacteroides proteoliticus DSM5265 obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH according to the procedures described in Example 1-(3). Both chromosomal DNAs were digested with EcoRI, PstI, HindIII, XbaI and SacI, respectively, which were subjected to 1% agarose gel electrophoresis, followed by southern hybridization according to the procedures described in Example 1-(5). As a probe, ^{32}P-labeled DNA probe prepared in Example 3-(1) was used. A membrane was washed at 37 °C in 2 × SSC finally containing 0.5% SDS, rinsed with 2 × SSC, and the autoradiogram was obtained. From this autoradiogram, a signal was recognized in an about 4.8 kb DNA fragment in a case of Staphylothermus marinus chromosomal DNA digested with PstI, and in an about 3.5 kb DNA fragment in a case of Thermobacteroides proteoliticus chromosomal DNA digested with XbaI, thus, indicating that a hyperthermostable protease gene which hybridizes with the protease PFUL gene was present in the Staphylothermus

marinus and Thermobacteroides proteoliticus chromosomal DNA.

Example 4

(1) Preparation of crude enzyme preparation of protease PFUS and TCES

Bacillus subtilis DB104 in which the plasmid pSTC3 containing the hyperthermostable protease gene of the present invention had been introduced (Bacillus subtilis DB104/pSTC3) was cultured in 5 ml of LB medium (trypton 10 g/liter, yeast extract 5 g/liter, NaCl 5 g/liter, pH 7.2) containing 10 μg/ml kanamycin at 37 °C for 8 hours. 250 ml of the similar medium was prepared in 1 liter Erlenmeyer flask, which was inoculated with 5 ml of the above culture to culture at 37 °C for 16 hours. Ammonium sulfate was added to a supernatant obtained by centrifugation of the culture to 75% saturation, and the resulted precipitates were recovered by centrifugation. The recovered precipitates were suspended in 4 ml of 20 mM Tris-HCl, pH 7.5, which was dialyzed against the same buffer, and the resulting dialysate was used as crude enzyme preparation (enzyme preparation TC-3).

Crude enzyme preparations were prepared from Bacillus subtilis DB104 in which the plasmid pSNP1 containing the hyperthermostable protease gene of the present invention was introduced (Bacillus subtilis DB104/pSNP1) or Bacillus subtilis DB104 in which the plasmid pSPT1 containing the hyperthermostable protease of the present invention, according to the procedures described above, and the preparations were designated NP-1 and PT-1, respectively.

These enzyme preparations were used to examine the protease activity by the enzyme activity detecting method using the SDS-polyacrylamide gel containing gelatin or by the other activity detecting methods.

(2) Preparation of purified enzyme preparation of protease PFUS

Two tubes containing 5 ml of LB medium containing 10 μl/ml kanamycin were inoculated with Bacillus subtilis DB104 in which the plasmid pNAPS1 containing the hyperthermostable protease gene of the present invention obtained in Example 2-(18) was introduced (Bacillus subtilis DB104/pNAPS1), followed by cultivation at 37 °C for 7 hours with shaking. Six Erlenmeyer flasks of 500 ml volume, each containing 120 ml of the similar medium, were prepared, and each flask was inoculated with 1 ml of the above culture, followed by cultivation at 37 °C for 17 hours with shaking. The culture was centrifuged to obtain the cells and a culture supernatant.

The cells were suspended in 15 ml of 50 mM Tris-HCl, pH 7.5, and 30 mg of lysozyme (manufactured by Sigma) was added thereto, followed by digestion at 37 °C for 1.5 hours. The digestion solution was heat-treated at 95 °C for 15 minutes, followed by centrifugation to collect a supernatant. To 12 ml of the resulting supernatant was added 4 ml of an saturated ammonium sulfate solution, which was filtrated using 0.45 μm filter unit (Sterivex HV, manufactured by Millipore), and the filtrate was loaded onto the POROS PH column (4.6 mm × 150 mm: manufactured by PerSeptive Biosystems) equilibrated with 25 mM Tris-HCl, pH 7.5 containing ammonium sulfate at 25% saturation. The column was washed with the buffer used for equilibration, the gradient elution was performed by lowering the concentration of ammonium sulfate from 25% saturation to 0% saturation and at the same time increasing the concentration of acetonitrile from 0% to 20% to elute the PFUS protease, to obtain the purified enzyme preparation NAPS-1.

750 ml of the culture supernatant was dialyzed against 25 mM Tris-HCl, pH 8.0 and adsorbed onto Econo-Pack Q cartridge (manufactured by BioRad) equilibrated with the same buffer. Then, the adsorbed enzyme was eluted with a linear gradient of 0 to 1.5 M NaCl. The resulting active fraction was heat-treated at 95 °C for 1 hour, and an 1/3 volume of a saturated ammonium sulfate solution was added thereto. After the filtration was carried out using a 0.45 μm filter unit (Sterivex HV), the filtrate was loaded onto the POROS PH column (4.6 mm × 150 mm) equilibrated with 25 mM Tris-HCl, pH 7.5 containing ammonium sulfate at 25% saturation. The PFUS protease absorbed onto the column was eluted according to the procedures as in the enzyme preparation NAPS-1 to obtain the purified enzyme preparation NAPS-1.

To an appropriate amount of the purified enzyme preparation NAPS-1 or NAPS-1S was added trichloroacetic acid to the final concentration of 8.3% to precipitate the proteins in the enzyme preparation, which were recovered by centrifugation. The recovered precipitated protein were dissolved in a distilled water, an 1/4 amount of a sample buffer (50 mM Tris-HCl, pH 7.5, 5% SDS, 5% 2-mercaptoethanol, 0.005% Bromophenol Blue, 50% glycerol) was added thereto, which was treated at 100 °C for 5 minutes and subjected to electrophoresis using 0.1% SDS-10% polyacrylamide gel. After run, the gel was stained in 2.5% Coomassie Brilliant Blue R-250, 25% ethanol, and 10% acetic acid for 30 minutes, transferred in 25% methanol, and 7% acetic acid and the excess dye was removed over 3 to 15 hours. Both enzyme preparations NAPS-1 and NAPS-1S showed a single band, and a molecular weight deduced from migrated distance was about 4.5 kDa.

(3) Sequencing of N-terminal of mature protease PUFS

The purified enzyme preparation NAPS-1 prepared in Example 4-(2) was subjected to electrophoresis using 0.1%

SDS-10% polyacrylamide gel, and the proteins on the gel was blotted onto a PVDF membrane (manufactured by Millipore) using Semidry Blotter (manufactured by Nihon Eido). Blotting was carried out according to a method described in Electrophoresis, volume 11, page 573-580 (1990). After blotting, the membrane was stained with a solution of 1% Coomassie Brilliant Blue R-250, in 50% methanol, and destained with a 60% methanol solution. A part of the membrane which had been stained was cut off, followed by sequencing of the N-terminal amino acid sequence by the automated Edman degradation using G1000A protein sequencer (manufactured by Hewlette Packard). SEQ ID No. 42 shows the resultant N-terminal amino acid sequence.

SEQUENCE LISTING

(1) GENERAL INFORMATION:
(i) APPLICANT:
(A) NAME: Takara Shuzo Co., Ltd.
(B) STREET: 609, Takenaka-cho, Fushimi-ku
(C) CITY: Kyoto-shi, Kyoto
(E) COUNTRY: Japan
(F) ZIP: 612

(ii) TITLE OF INVENTION: Hyperthermostable protease gene

(iii) NUMBER OF SEQUENCES: 42

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: 3.5" Diskette, 1.44 Mb
(B) COMPUTER: IBM PS/2 Model 50Z or 55SX
(C) OPERATING SYSTEM: MS-DOS (Version 5.0)
(D) SOFTWARE: Microsoft Word

(v) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: EP 96937514.6

(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: PCT/JP96/03253
(B) FILING DATE: 7. November 1996

(2) INFORMATION FOR SEQ ID NO:1
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 659
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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(2) INFORMATION FOR SEQ ID NO:2
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 1977
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: genomic DNA
(vi) ORIGINAL SOURCE: Thermococcus celer
(B) STRAIN: DSM2476
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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(2) INFORMATION FOR SEQ ID NO:3
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 522
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(ix) FEATURE: Xaa at 428 position is Gly or Val.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

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(2) INFORMATION FOR SEQ ID NO:4
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 1566
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: genomic DNA
(vi) ORIGINAL SOURCE: Pyrococcus furiosus
(B) STRAIN: DSM3638
(ix) FEATURE: N at 1283 position is G or T.
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

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ATCGTAAAGC CAGATGAAAT AGCCGATATA GCCTACGGTG CAGGTAGGGT TAATGCATAC  900
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GACTACTCTT ACACCGCCTA CTATGGATTC GAAAAGGTTG GTTATTACAA CCCAACTGAT  1140
GGAACATGGA CAATTAAGGT TGTAAGCTAC AGCGGAAGTG CAAACTATCA AGTAGATGTG  1200
GTAAGTGATG GTTCCCTTTC ACAGCCTGGA AGTTCACCAT CTCCACAACC AGAACCAACA  1260
GTAGACGCAA AGACGTTCCA AGNATCCGAT CACTACTACT ATGACAGGAG CGACACCTTT  1320
ACAATGACCG TTAACTCTGG GGCTACAAAG ATTACTGGAG ACCTAGTGTT TGACACAAGC  1380
TACCATGATC TTGACCTTTA CCTCTACGAT CCTAACCAGA AGCTTGTAGA TAGATCGGAG  1440
AGTCCCAACA GCTACGAACA CGTAGAATAC TTAACCCCCG CCCCAGGAAC CTGGTACTTC  1500
CTAGTATATG CCTACTACAC TTACGGTTGG GCTTACTACG AGCTGACGGC TAAAGTTTAT  1560
TATGGC                                                             1566
```

(2) INFORMATION FOR SEQ ID NO:5

(i) SEQUENCE CHARACTERISTICS

(A) LENGTH: 659

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
Met Lys Gly Leu Lys Ala Leu Ile Leu Val Ile Leu Val Leu Gly
  5                  10                  15
Leu Val Val Gly Ser Val Ala Ala Ala Pro Glu Lys Lys Val Glu
            20                  25                  30
Gln Val Arg Asn Val Glu Lys Asn Tyr Gly Leu Leu Thr Pro Gly
        35                  40                  45
Leu Phe Arg Lys Ile Gln Lys Leu Asn Pro Asn Glu Glu Ile Ser
    50                  55                  60
Thr Val Ile Val Phe Glu Asn His Arg Glu Lys Glu Ile Ala Val
65                  70                  75
Arg Val Leu Glu Leu Met Gly Ala Lys Val Arg Tyr Val Tyr His
            80                  85                  90
Ile Ile Pro Ala Ile Ala Ala Asp Leu Lys Val Arg Asp Leu Leu
        95                  100                 105
Val Ile Ser Gly Leu Thr Gly Gly Lys Ala Lys Leu Ser Gly Val
    110                 115                 120
Arg Phe Ile Gln Glu Asp Tyr Lys Val Thr Val Ser Ala Glu Leu
125                 130                 135
Glu Gly Leu Asp Glu Ser Ala Ala Gln Val Met Ala Thr Tyr Val
            140                 145                 150
Trp Asn Leu Gly Tyr Asp Gly Ser Gly Ile Thr Ile Gly Ile Ile
        155                 160                 165
Asp Thr Gly Ile Asp Ala Ser His Pro Asp Leu Gln Gly Lys Val
    170                 175                 180
Ile Gly Trp Val Asp Phe Val Asn Gly Arg Ser Tyr Pro Tyr Asp
185                 190                 195
Asp His Gly His Gly Thr His Val Ala Ser Ile Ala Ala Gly Thr
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                                200                 205                 210
Gly Ala Ala Ser Asn Gly Lys Tyr Lys Gly Met Ala Pro Gly Ala
                215                 220                 225
Lys Leu Ala Gly Ile Lys Val Leu Gly Ala Asp Gly Ser Gly Ser
                230                 235                 240
Ile Ser Thr Ile Ile Lys Gly Val Glu Trp Ala Val Asp Asn Lys
                245                 250                 255
Asp Lys Tyr Gly Ile Lys Val Ile Asn Leu Ser Leu Gly Ser Ser
                260                 265                 270
Gln Ser Ser Asp Gly Thr Asp Ser Leu Ser Gln Ala Val Asn Asn
                275                 280                 285
Ala Trp Asp Ala Gly Ile Val Val Cys Val Ala Ala Gly Asn Ser
                290                 295                 300
Gly Pro Asn Thr Tyr Thr Val Gly Ser Pro Ala Ala Ala Ser Lys
                305                 310                 315
Val Ile Thr Val Gly Ala Val Asp Ser Asn Asp Asn Ile Ala Ser
                320                 325                 330
Phe Ser Ser Arg Gly Pro Thr Ala Asp Gly Arg Leu Lys Pro Glu
                335                 340                 345
Val Val Ala Pro Gly Val Asp Ile Ile Ala Pro Arg Ala Ser Gly
                350                 355                 360
Thr Ser Met Gly Thr Pro Ile Asn Asp Tyr Tyr Thr Lys Ala Ser
                365                 370                 375
Gly Thr Ser Met Ala Thr Pro His Val Ser Gly Val Gly Ala Leu
                380                 385                 390
Ile Leu Gln Ala His Pro Ser Trp Thr Pro Asp Lys Val Lys Thr
                395                 400                 405
Ala Leu Ile Glu Thr Ala Asp Ile Val Ala Pro Lys Glu Ile Ala
                410                 415                 420
Asp Ile Ala Tyr Gly Ala Gly Arg Val Asn Val Tyr Lys Ala Ile
                425                 430                 435
Lys Tyr Asp Asp Tyr Ala Lys Leu Thr Phe Thr Gly Ser Val Ala
                440                 445                 450
Asp Lys Gly Ser Ala Thr His Thr Phe Asp Val Ser Gly Ala Thr
                455                 460                 465
Phe Val Thr Ala Thr Leu Tyr Trp Asp Thr Gly Ser Ser Asp Ile
                470                 475                 480
Asp Leu Tyr Leu Tyr Asp Pro Asn Gly Asn Glu Val Asp Tyr Ser
                485                 490                 495
Tyr Thr Ala Tyr Tyr Gly Phe Glu Lys Val Gly Tyr Tyr Asn Pro
                500                 505                 510
Thr Ala Gly Thr Trp Thr Val Lys Val Val Ser Tyr Lys Gly Ala
                515                 520                 525
Ala Asn Tyr Gln Val Asp Val Val Ser Asp Gly Ser Leu Ser Gln
                530                 535                 540
Ser Gly Gly Gly Asn Pro Asn Pro Asn Pro Asn Pro Asn Pro Thr
```

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                545                 550                 555
Pro Thr Thr Asp Thr Gln Thr Phe Thr Gly Ser Val Asn Asp Tyr
                560                 565                 570
Trp Asp Thr Ser Asp Thr Phe Thr Met Asn Val Asn Ser Gly Ala
                575                 580                 685
Thr Lys Ile Thr Gly Asp Leu Thr Phe Asp Thr Ser Tyr Asn Asp
                590                 595                 600
Leu Asp Leu Tyr Leu Tyr Asp Pro Asn Gly Asn Leu Val Asp Arg
                605                 610                 615
Ser Thr Ser Ser Asn Ser Tyr Glu His Val Glu Tyr Ala Asn Pro
                620                 625                 630
Ala Pro Gly Thr Trp Thr Phe Leu Val Tyr Ala Tyr Ser Thr Tyr
                635                 640                 645
Gly Trp Ala Asp Tyr Gln Leu Lys Ala Val Val Tyr Tyr Gly
                650                 655
```

(2) INFORMATION FOR SEQ ID NO:6
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 1977
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: genomic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
ATGAAGGGGC TGAAAGCTCT CATATTAGTG ATTTTAGTTC TAGGTTTGGT AGTAGGGAGC   60
GTAGCGGCAG CTCCAGAGAA GAAAGTTGAA CAAGTAAGAA ATGTTGAGAA GAACTATGGT  120
CTGCTAACGC CAGGACTGTT CAGAAAAATT CAAAAATTGA ATCCTAACGA GGAAATCAGC  180
ACAGTAATTG TATTTGAAAA CCATAGGGAA AAAGAAATTG CAGTAAGAGT TCTTGAGTTA  240
ATGGGTGCAA AAGTTAGGTA TGTGTACCAT ATTATACCCG CAATAGCTGC CGATCTTAAG  300
GTTAGAGACT TACTAGTCAT CTCAGGTTTA ACAGGGGGTA AAGCTAAGCT TTCAGGTGTT  360
AGGTTTATCC AGGAAGACTA CAAAGTTACA GTTTCAGCAG AATTAGAAGG ACTGGATGAG  420
TCTGCAGCTC AAGTTATGGC AACTTACGTT TGGAACTTGG GATATGATGG TTCTGGAATC  480
ACAATAGGAA TAATTGACAC TGGAATTGAC GCTTCTCATC CAGATCTCCA AGGAAAAGTA  540
ATTGGGTGGG TAGATTTTGT CAATGGTAGG AGTTATCCAT ACGATGACCA TGGACATGGA  600
ACTCATGTAG CTTCAATAGC AGCTGGTACT GGAGCAGCAA GTAATGGCAA GTACAAGGGA  660
ATGGCTCCAG GAGCTAAGCT GGCGGGAATT AAGGTTCTAG GTGCCGATGG TTCTGGAAGC  720
ATATCTACTA TAATTAAGGG AGTTGAGTGG GCCGTTGATA ACAAAGATAA GTACGGAATT  780
AAGGTCATTA ATCTTTCTCT TGGTTCAAGC CAGAGCTCCG ACGGAACCGA CTCCCTCAGT  840
CAGGCCGTCA ACAACGCCTG GGACGCCGGT ATAGTAGTCT GCGTCGCCGC CGGCAACAGC  900
GGGCCGAACA CCTACACCGT CGGCTCACCC GCCGCCGCGA GCAAGGTCAT AACCGTCGGT  960
GCAGTTGACA GCAACGACAA CATCGCCAGC TTCTCCAGCA GGGGACCGAC CGCGGACGGA 1020
AGGCTCAAGC CGGAAGTCGT CGCCCCCGGC GTTGACATCA TAGCCCCGCG CGCCAGCGGA 1080
ACCAGCATGG GCACCCCGAT AAACGACTAC TACACCAAGG CCTCTGGAAC CAGCATGGCC 1140
ACCCCGCACG TTTCGGGCGT TGGCGCGCTC ATCCTCCAGG CCCACCCGAG CTGGACCCCG 1200
GACAAGGTGA AGACCGCCCT CATCGAGACC GCCGACATAG TCGCCCCCAA GGAGATAGCG 1260
GACATCGCCT ACGGTGCGGG TAGGGTGAAC GTCTACAAGG CCATCAAGTA CGACGACTAC 1320
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GCCAAGCTCA CCTTCACCGG CTCCGTCGCC GACAAGGGAA GCGCCACCCA CACCTTCGAC 1380
GTCAGCGGCG CCACCTTCGT GACCGCCACC CTCTACTGGG ACACGGGCTC GAGCGACATC 1440
GACCTCTACC TCTACGACCC CAACGGGAAC GAGGTTGACT ACTCCTACAC CGCCTACTAC 1500
GGCTTCGAGA AGGTCGGCTA CTACAACCCG ACCGCCGGAA CCTGGACGGT CAAGGTCGTC 1560
AGCTACAAGG GCGCGGCGAA CTACCAGGTC GACGTCGTCA GCGACGGGAG CCTCAGCCAG 1620
TCCGGCGGCG GCAACCCGAA TCCAAACCCC AACCCGAACC CAACCCCGAC CACCGACACC 1680
CAGACCTTCA CCGGTTCCGT TAACGACTAC TGGGACACCA GCGACACCTT CACCATGAAC 1740
GTCAACAGCG GTGCCACCAA GATAACCGGT GACCTGACCT TCGATACTTC CTACAACGAC 1800
CTCGACCTCT ACCTCTACGA CCCCAACGGC AACCTCGTTG ACAGGTCCAC GTCGAGCAAC 1860
AGCTACGAGC ACGTCGAGTA CGCCAACCCC GCCCCGGGAA CCTGGACGTT CCTCGTCTAC 1920
GCCTACAGCA CCTACGGCTG GGCGGACTAC CAGCTCAAGG CCGTCGTCTA CTACGGG 1977

(2) INFORMATION FOR SEQ ID NO:7
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 4765
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: genomic DNA
(vi) ORIGINAL SOURCE: Pyrococcus furiosus
(B) STRAIN: DSM3638
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

TTTAAATTAT AAGATATAAT CACTCCGAGT GATGAGTAAG ATACATCATT ACAGTCCCAA 60
AATGTTTATA ATTGGAACGC AGTGAATATA CAAAATGAAT ATAACCTCGG AGGTGACTGT 120
AGAATGAATA AGAAGGGACT TACTGTGCTA TTTATAGCGA TAATGCTCCT TTCAGTAGTT 180
CCAGTGCACT TTGTGTCCGC AGAAACACCA CCGGTTAGTT CAGAAAATTC AACAACTTCT 240
ATACTCCCTA ACCAACAAGT TGTGACAAAA GAAGTTTCAC AAGCGGCGCT TAATGCTATA 300
ATGAAAGGAC AACCCAACAT GGTTCTTATA ATCAAGACTA AGGAAGGCAA ACTTGAAGAG 360
GCAAAAACCG AGCTTGAAAA GCTAGGTGCA GAGATTCTTG ACGAAAATAG AGTTCTTAAC 420
ATGTTGCTAG TTAAGATTAA GCCTGAGAAA GTTAAAGAGC TCAACTATAT CTCATCTCTT 480
GAAAAAGCCT GGCTTAACAG AGAAGTTAAG CTTTCCCCTC CAATTGTCGA AAAGGACGTC 540
AAGACTAAGG AGCCCTCCCT AGAACCAAAA ATGTATAACA GCACCTGGGT AATTAATGCT 600
CTCCAGTTCA TCCAGGAATT TGGATATGAT GGTAGTGGTG TTGTTGTTGC AGTACTTGAC 660
ACGGGAGTTG ATCCGAACCA TCCTTTCTTG AGCATAACTC CAGATGGACG CAGGAAAATT 720
ATAGAATGGA AGGATTTTAC AGACGAGGGA TTCGTGGATA CATCATTCAG CTTTAGCAAG 780
GTTGTAAATG GGACTCTTAT AATTAACACA ACATTCCAAG TGGCCTCAGG TCTCACGCTG 840
AATGAATCGA CAGGACTTAT GGAATACGTT GTTAAGACTG TTTACGTGAG CAATGTGACC 900
ATTGGAAATA TCACTTCTGC TAATGGCATC TATCACTTCG GCCTGCTCCC AGAAAGATAC 960
TTCGACTTAA ACTTCGATGG TGATCAAGAG GACTTCTATC CTGTCTTATT AGTTAACTCC 1020
ACTGGCAATG GTTATGACAT TGCATATGTG GATACTGACC TTGACTACGA CTTCACCGAC 1080
GAAGTTCCAC TTGGCCAGTA CAACGTTACT TATGATGTTG CTGTTTTTAG CTACTACTAC 1140
GGTCCTCTCA ACTACGTGCT TGCAGAAATA GATCCTAACG GAGAATATGC AGTATTTGGG 1200
TGGGATGGTC ACGGTCACGG AACTCACGTA GCTGGAACTG TTGCTGGTTA CGACAGCAAC 1260
AATGATGCTT GGGATTGGCT CAGTATGTAC TCTGGTGAAT GGGAAGTGTT CTCAAGACTC 1320
TATGGTTGGG ATTATACGAA CGTTACCACA GACACCGTGC AGGGTGTTGC TCCAGGTGCC 1380
CAAATAATGG CAATAAGAGT TCTTAGGAGT GATGGACGGG GTAGCATGTG GGATATTATA 1440

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GAAGGTATGA CATACGCAGC AACCCATGGT GCAGACGTTA TAAGCATGAG TCTCGGTGGA  1500
AATGCTCCAT ACTTAGATGG TACTGATCCA GAAAGCGTTG CTGTGGATGA GCTTACCGAA  1560
AAGTACGGTG TTGTATTCGT AATAGCTGCA GGAAATGAAG GTCCTGGCAT TAACATCGTT  1620
GGAAGTCCTG GTGTTGCAAC AAAGGCAATA ACTGTTGGAG CTGCTGCAGT GCCCATTAAC  1680
GTTGGAGTTT ATGTTTCCCA AGCACTTGGA TATCCTGATT ACTATGGATT CTATTACTTC  1740
CCCGCCTACA CAAACGTTAG AATAGCATTC TTCTCAAGCA GAGGGCCGAG AATAGATGGT  1800
GAAATAAAAC CCAATGTAGT GGCTCCAGGT TACGGAATTT ACTCATCCCT GCCGATGTGG  1860
ATTGGCGGAG CTGACTTCAT GTCTGGAACT TCGATGGCTA CTCCACATGT CAGCGGTGTC  1920
GTTGCACTCC TCATAAGCGG GGCAAAGGCC GAGGGAATAT ACTACAATCC AGATATAATT  1980
AAGAAGGTTC TTGAGAGCGG TGCAACCTGG CTTGAGGGAG ATCCATATAC TGGGCAGAAG  2040
TACACTGAGC TTGACCAAGG TCATGGTCTT GTTAACGTTA CCAAGTCCTG GGAAATCCTT  2100
AAGGCTATAA ACGGCACCAC TCTCCCAATT GTTGATCACT GGGCAGACAA GTCCTACAGC  2160
GACTTTGCGG AGTACTTGGG TGTGGACGTT ATAAGAGGTC TCTACGCAAG GAACTCTATA  2220
CCTGACATTG TCGAGTGGCA CATTAAGTAC GTAGGGGACA CGGAGTACAG AACTTTTGAG  2280
ATCTATGCAA CTGAGCCATG GATTAAGCCT TTTGTCAGTG GAAGTGTAAT TCTAGAGAAC  2340
AATACCGAGT TTGTCCTTAG GGTGAAATAT GATGTAGAGG GTCTTGAGCC AGGTCTCTAT  2400
GTTGGAAGGA TAATCATTGA TGATCCAACA ACGCCAGTTA TTGAAGACGA GATCTTGAAC  2460
ACAATTGTTA TTCCCGAGAA GTTCACTCCT GAGAACAATT ACACCCTCAC CTGGTATGAT  2520
ATTAATGGTC CAGAAATGGT GACTCACCAC TTCTTCACTG TGCCTGAGGG AGTGGACGTT  2580
CTCTACGCGA TGACCACATA CTGGGACTAC GGTCTGTACA GACCAGATGG AATGTTTGTG  2640
TTCCCATACC AGCTAGATTA TCTTCCCGCT GCAGTCTCAA ATCCAATGCC TGGAAACTGG  2700
GAGCTAGTAT GGACTGGATT TAACTTTGCA CCCCTCTATG AGTCGGGCTT CCTTGTAAGG  2760
ATTTACGGAG TAGAGATAAC TCCAAGCGTT TGGTACATTA ACAGGACATA CCTTGACACT  2820
AACACTGAAT TCTCAATTGA ATTCAATATT ACTAACATCT ATGCCCCAAT TAATGCAACT  2880
CTAATCCCCA TTGGCCTTGG AACCTACAAT GCGAGCGTTG AAAGCGTTGG TGATGGAGAG  2940
TTCTTCATAA AGGGCATTGA AGTTCCTGAA GGCACCGCAG AGTTGAAGAT TAGGATAGGC  3000
AACCCAAGTG TTCCGAATTC AGATCTAGAC TTGTACCTTT ATGACAGTAA AGGCAATTTA  3060
GTGGCCTTAG ATGGAAACCC AACAGCAGAA GAAGAGGTTG TAGTTGAGTA TCCTAAGCCT  3120
GGAGTTTATT CAATAGTAGT ACATGGTTAC AGCGTCAGGG ACGAAAATGG TAATCCAACG  3180
ACAACCACCT TTGACTTAGT TGTTCAAATG ACCCTTGATA ATGGAAACAT AAAGCTTGAC  3240
AAAGACTCGA TTATTCTTGG AAGCAATGAA AGCGTAGTTG TAACTGCAAA CATAACAATT  3300
GATAGAGATC ATCCTACAGG AGTATACTCT GGTATCATAG AGATTAGAGA TAATGAGGTC  3360
TACCAGGATA CAAATACTTC AATTGCGAAA ATACCCATAA CTTTGGTAAT TGACAAGGCG  3420
GACTTTGCCG TTGGTCTCAC ACCAGCAGAG GGAGTACTTG GAGAGGCTAG AAATTACACT  3480
CTAATTGTAA AGCATGCCCT AACACTAGAG CCTGTGCCAA ATGCTACAGT GATTATAGGA  3540
AACTACACCT ACCTCACAGA CGAAAACGGT ACAGTGACAT TCACGTATGC TCCAACTAAG  3600
TTAGGCAGTG ATGAAATCAC AGTCATAGTT AAGAAAGAGA ACTTCAACAC ATTAGAGAAG  3660
ACCTTCCAAA TCACAGTATC AGAGCCTGAA ATAACTGAAG AGGACATAAA TGAGCCCAAG  3720
CTTGCAATGT CATCACCAGA AGCAAATGCT ACCATAGTAT CAGTTGAGAT GGAGAGTGAG  3780
GGTGGCGTTA AAAAGACAGT GACAGTGGAA ATAACTATAA ACGGAACCGC TAATGAGACT  3840
GCAACAATAG TGGTTCCTGT TCCTAAGAAG GCCGAAAACA TCGAGGTAAG TGGAGACCAC  3900
GTAATTTCCT ATAGTATAGA GGAAGGAGAG TACGCCAAGT ACGTTATAAT TACAGTGAAG  3960
TTTGCATCAC CTGTAACAGT AACTGTTACT TACACTATCT ATGCTGGCCC AAGAGTCTCA  4020
ATCTTGACAC TTAACTTCCT TGGCTACTCA TGGTACAGAC TATATTCACA GAAGTTTGAC  4080
GAATTGTACC AAAAGGCCCT TGAATTGGGA GTGGACAACG AGACATTAGC TTTAGCCCTC  4140
AGCTACCATG AAAAAGCCAA AGAGTACTAC GAAAAGGCCC TTGAGCTTAG CGAGGGTAAC  4200
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ATAATCCAAT ACCTTGGAGA CATAAGACTA TTACCTCCAT TAAGACAGGC ATACATCAAT  4260
GAAATGAAGG CAGTTAAGAT ACTGGAAAAG GCCATAGAAG AATTAGAGGG TGAAGAGTAA  4320
TCTCCAATTT TTCCCACTTT TTCTTTTATA ACATTCCAAG CCTTTTCTTA GCTTCTTCGC  4380
TCATTCTATC AGGAGTCCAT GGAGGATCAA AGGTAAGTTC AACCTCCACA TCTCTTACTC  4440
CTGGGATTTC GAGTACTTTC TCCTCTACAG CTCTAAGAAG CCAGAGAGTT AAAGGACACC  4500
CAGGAGTTGT CATTGTCATC TTTATATATA CCGTTTTGTC AGGATTAATC TTTAGCTCAT  4560
AAATTAATCC AAGGTTTACA ACATCCATCC CAATTTCTGG GTCGATAACC TCCTTTAGCT  4620
TTTCCAGAAT CATTTCTTCA GTAATTTCAA GGTTCTCATC TTTGGTTTCT CTCACAAACC  4680
CAATTTCAAC CTGCCTGATA CCTTCTAACT CCCTAAGCTT GTTATATATC TCCAAAAGAG  4740
TGGCATCATC AATTTTCTCT TTAAA 3'                                     4765
```

(2) INFORMATION FOR SEQ ID NO:8
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 1398
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

```
Met Asn Lys Lys Gly Leu Thr Val Leu Phe Ile Ala Ile Met Leu
 1               5                  10                  15
Leu Ser Val Val Pro Val His Phe Val Ser Ala Glu Thr Pro Pro
            20                  25                  30
Val Ser Ser Glu Asn Ser Thr Thr Ser Ile Leu Pro Asn Gln Gln
        35                  40                  45
Val Val Thr Lys Glu Val Ser Gln Ala Ala Leu Asn Ala Ile Met
    50                  55                  60
Lys Gly Gln Pro Asn Met Val Leu Ile Ile Lys Thr Lys Glu Gly
65                  70                  75
Lys Leu Glu Glu Ala Lys Thr Glu Leu Glu Lys Leu Gly Ala Glu
                80                  85                  90
Ile Leu Asp Glu Asn Arg Val Leu Asn Met Leu Leu Val Lys Ile
            95                  100                 105
Lys Pro Glu Lys Val Lys Glu Leu Asn Tyr Ile Ser Ser Leu Glu
        110                 115                 120
Lys Ala Trp Leu Asn Arg Glu Val Lys Leu Ser Pro Pro Ile Val
    125                 130                 135
Glu Lys Asp Val Lys Thr Lys Glu Pro Ser Leu Glu Pro Lys Met
140                 145                 150
Tyr Asn Ser Thr Trp Val Ile Asn Ala Leu Gln Phe Ile Gln Glu
155                 160                 165
Phe Gly Tyr Asp Gly Ser Gly Val Val Val Ala Val Leu Asp Thr
            170                 175                 180
Gly Val Asp Pro Asn His Pro Phe Leu Ser Ile Thr Pro Asp Gly
        185                 190                 195
Arg Arg Lys Ile Ile Glu Trp Lys Asp Phe Thr Asp Glu Gly Phe
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                    200                 205                 210
Val Asp Thr Ser Phe Ser Phe Ser Lys Val Val Asn Gly Thr Leu
                215                 220                 225
Ile Ile Asn Thr Thr Phe Gln Val Ala Ser Gly Leu Thr Leu Asn
                230                 235                 240
Glu Ser Thr Gly Leu Met Glu Tyr Val Val Lys Thr Val Tyr Val
                245                 250                 255
Ser Asn Val Thr Ile Gly Asn Ile Thr Ser Ala Asn Gly Ile Tyr
                260                 265                 270
His Phe Gly Leu Leu Pro Glu Arg Tyr Phe Asp Leu Asn Phe Asp
                275                 280                 285
Gly Asp Gln Glu Asp Phe Tyr Pro Val Leu Leu Val Asn Ser Thr
                290                 295                 300
Gly Asn Gly Tyr Asp Ile Ala Tyr Val Asp Thr Asp Leu Asp Tyr
                305                 310                 315
Asp Phe Thr Asp Glu Val Pro Leu Gly Gln Tyr Asn Val Thr Tyr
                320                 325                 330
Asp Val Ala Val Phe Ser Tyr Tyr Tyr Gly Pro Leu Asn Tyr Val
                335                 340                 345
Leu Ala Glu Ile Asp Pro Asn Gly Glu Tyr Ala Val Phe Gly Trp
                350                 355                 360
Asp Gly His Gly His Gly Thr His Val Ala Gly Thr Val Ala Gly
                365                 370                 375
Tyr Asp Ser Asn Asn Asp Ala Trp Asp Trp Leu Ser Met Tyr Ser
                380                 385                 390
Gly Glu Trp Glu Val Phe Ser Arg Leu Tyr Gly Trp Asp Tyr Thr
                395                 400                 405
Asn Val Thr Thr Asp Thr Val Gln Gly Val Ala Pro Gly Ala Gln
                410                 415                 420
Ile Met Ala Ile Arg Val Leu Arg Ser Asp Gly Arg Gly Ser Met
                425                 430                 435
Trp Asp Ile Ile Glu Gly Met Thr Tyr Ala Ala Thr His Gly Ala
                440                 445                 450
Asp Val Ile Ser Met Ser Leu Gly Gly Asn Ala Pro Tyr Leu Asp
                455                 460                 465
Gly Thr Asp Pro Glu Ser Val Ala Val Asp Glu Leu Thr Glu Lys
                470                 475                 480
Tyr Gly Val Val Phe Val Ile Ala Ala Gly Asn Glu Gly Pro Gly
                485                 490                 495
Ile Asn Ile Val Gly Ser Pro Gly Val Ala Thr Lys Ala Ile Thr
                500                 505                 510
Val Gly Ala Ala Ala Val Pro Ile Asn Val Gly Val Tyr Val Ser
                515                 520                 525
Gln Ala Leu Gly Tyr Pro Asp Tyr Tyr Gly Phe Tyr Tyr Phe Pro
                530                 535                 540
Ala Tyr Thr Asn Val Arg Ile Ala Phe Phe Ser Ser Arg Gly Pro
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                545                 550                 555
Arg Ile Asp Gly Glu Ile Lys Pro Asn Val Val Ala Pro Gly Tyr
            560                 565                 570
Gly Ile Tyr Ser Ser Leu Pro Met Trp Ile Gly Gly Ala Asp Phe
        575                 580                 585
Met Ser Gly Thr Ser Met Ala Thr Pro His Val Ser Gly Val Val
    590                 595                 600
Ala Leu Leu Ile Ser Gly Ala Lys Ala Glu Gly Ile Tyr Tyr Asn
605                 610                 615
Pro Asp Ile Ile Lys Lys Val Leu Glu Ser Gly Ala Thr Trp Leu
620                 625                 630
Glu Gly Asp Pro Tyr Thr Gly Gln Lys Tyr Thr Glu Leu Asp Gln
635                 640                 645
Gly His Gly Leu Val Asn Val Thr Lys Ser Trp Glu Ile Leu Lys
650                 655                 660
Ala Ile Asn Gly Thr Thr Leu Pro Ile Val Asp His Trp Ala Asp
665                 670                 675
Lys Ser Tyr Ser Asp Phe Ala Glu Tyr Leu Gly Val Asp Val Ile
680                 685                 690
Arg Gly Leu Tyr Ala Arg Asn Ser Ile Pro Asp Ile Val Glu Trp
695                 700                 705
His Ile Lys Tyr Val Gly Asp Thr Glu Tyr Arg Thr Phe Glu Ile
710                 715                 720
Tyr Ala Thr Glu Pro Trp Ile Lys Pro Phe Val Ser Gly Ser Val
725                 730                 735
Ile Leu Glu Asn Asn Thr Glu Phe Val Leu Arg Val Lys Tyr Asp
740                 745                 750
Val Glu Gly Leu Glu Pro Gly Leu Tyr Val Gly Arg Ile Ile Ile
755                 760                 765
Asp Asp Pro Thr Thr Pro Val Ile Glu Asp Glu Ile Leu Asn Thr
770                 775                 780
Ile Val Ile Pro Glu Lys Phe Thr Pro Glu Asn Asn Tyr Thr Leu
785                 790                 795
Thr Trp Tyr Asp Ile Asn Gly Pro Glu Met Val Thr His His Phe
800                 805                 810
Phe Thr Val Pro Glu Gly Val Asp Val Leu Tyr Ala Met Thr Thr
815                 820                 825
Tyr Trp Asp Tyr Gly Leu Tyr Arg Pro Asp Gly Met Phe Val Phe
830                 835                 840
Pro Tyr Gln Leu Asp Tyr Leu Pro Ala Ala Val Ser Asn Pro Met
845                 850                 855
Pro Gly Asn Trp Glu Leu Val Trp Thr Gly Phe Asn Phe Ala Pro
860                 865                 870
Leu Tyr Glu Ser Gly Phe Leu Val Arg Ile Tyr Gly Val Glu Ile
875                 880                 885
Thr Pro Ser Val Trp Tyr Ile Asn Arg Thr Tyr Leu Asp Thr Asn
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890 895 900
Thr Glu Phe Ser Ile Glu Phe Asn Ile Thr Asn Ile Tyr Ala Pro
905 910 915
Ile Asn Ala Thr Leu Ile Pro Ile Gly Leu Gly Thr Tyr Asn Ala
920 925 930
Ser Val Glu Ser Val Gly Asp Gly Glu Phe Phe Ile Lys Gly Ile
935 940 945
Glu Val Pro Glu Gly Thr Ala Glu Leu Lys Ile Arg Ile Gly Asn
950 955 960
Pro Ser Val Pro Asn Ser Asp Leu Asp Leu Tyr Leu Tyr Asp Ser
965 970 975
Lys Gly Asn Leu Val Ala Leu Asp Gly Asn Pro Thr Ala Glu Glu
980 985 990
Glu Val Val Val Glu Tyr Pro Lys Pro Gly Val Tyr Ser Ile Val
995 1000 1005
Val His Gly Tyr Ser Val Arg Asp Glu Asn Gly Asn Pro Thr Thr
1010 1015 1020
Thr Thr Phe Asp Leu Val Val Gln Met Thr Leu Asp Asn Gly Asn
1025 1030 1035
Ile Lys Leu Asp Lys Asp Ser Ile Ile Leu Gly Ser Asn Glu Ser
1040 1045 1050
Val Val Val Thr Ala Asn Ile Thr Ile Asp Arg Asp His Pro Thr
1055 1060 1065
Gly Val Tyr Ser Gly Ile Ile Glu Ile Arg Asp Asn Glu Val Tyr
1070 1075 1080
Gln Asp Thr Asn Thr Ser Ile Ala Lys Ile Pro Ile Thr Leu Val
1085 1090 1095
Ile Asp Lys Ala Asp Phe Ala Val Gly Leu Thr Pro Ala Glu Gly
1100 1105 1110
Val Leu Gly Glu Ala Arg Asn Tyr Thr Leu Ile Val Lys His Ala
1115 1120 1125
Leu Thr Leu Glu Pro Val Pro Asn Ala Thr Val Ile Ile Gly Asn
1130 1135 1140
Tyr Thr Tyr Leu Thr Asp Glu Asn Gly Thr Val Thr Phe Thr Tyr
1145 1150 1155
Ala Pro Thr Lys Leu Gly Ser Asp Glu Ile Thr Val Ile Val Lys
1160 1165 1170
Lys Glu Asn Phe Asn Thr Leu Glu Lys Thr Phe Gln Ile Thr Val
1175 1180 1185
Ser Glu Pro Glu Ile Thr Glu Glu Asp Ile Asn Glu Pro Lys Leu
1190 1195 1200
Ala Met Ser Ser Pro Glu Ala Asn Ala Thr Ile Val Ser Val Glu
1205 1210 1215
Met Glu Ser Glu Gly Gly Val Lys Lys Thr Val Thr Val Glu Ile
1220 1225 1230
Thr Ile Asn Gly Thr Ala Asn Glu Thr Ala Thr Ile Val Val Pro

```
                1235                1240                1245
Val Pro Lys Lys Ala Glu Asn Ile Glu Val Ser Gly Asp His Val
                1250                1255                1260
Ile Ser Tyr Ser Ile Glu Glu Gly Glu Tyr Ala Lys Tyr Val Ile
                1265                1270                1275
Ile Thr Val Lys Phe Ala Ser Pro Val Thr Val Thr Val Thr Tyr
                1280                1285                1290
Thr Ile Tyr Ala Gly Pro Arg Val Ser Ile Leu Thr Leu Asn Phe
                1295                1300                1305
Leu Gly Tyr Ser Trp Tyr Arg Leu Tyr Ser Gln Lys Phe Asp Glu
                1310                1315                1320
Leu Tyr Gln Lys Ala Leu Glu Leu Gly Val Asp Asn Glu Thr Leu
                1325                1330                1335
Ala Leu Ala Leu Ser Tyr His Glu Lys Ala Lys Glu Tyr Tyr Glu
                1340                1345                1350
Lys Ala Leu Glu Leu Ser Glu Gly Asn Ile Ile Gln Tyr Leu Gly
                1355                1360                1365
Asp Ile Arg Leu Leu Pro Pro Leu Arg Gln Ala Tyr Ile Asn Glu
                1370                1375                1380
Met Lys Ala Val Lys Ile Leu Glu Lys Ala Ile Glu Glu Leu Glu
                1385                1390                1395
Gly Glu Glu
```

(2) INFORMATION FOR SEQ ID NO:9
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 35
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:
GGWWSDRRTG TTRRHGTHGC DGTDMTYGAC ACBGG 35

(2) INFORMATION FOR SEQ ID NO:10
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 32
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:
KSTCACGGAA CTCACGTDGC BGGMACDGTT GC 32

(2) INFORMATION FOR SEQ ID NO:11
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 33

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:
ASCMGCAACH GTKCCVGCHA CGTGAGTTCC GTG 33

(2) INFORMATION FOR SEQ ID NO:12
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 34
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:
CHCCGSYVAC RTGBGGAGWD GCCATBGAVG TDCC 34

(2) INFORMATION FOR SEQ ID NO:13
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 145
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (PCR fragment)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

```
A GTT GCG GTA ATT GAC ACG GGT ATA GAC GCG AAC CAC CCC GAT CTG      46
  Val Ala Val Ile Asp Thr Gly Ile Asp Ala Asn His Pro Asp Leu
                  5                  10                  15
  AAG GGC AAG GTC ATA GGC TGG TAC GAC GCC GTC AAC GGC AGG TCG      91
  Lys Gly Lys Val Ile Gly Trp Tyr Asp Ala Val Asn Gly Arg Ser
              20                  25                  30
  ACC CCC TAC GAT GAC CAG GGA CAC GGA ACT CAC GTN GCN GGA ACN     136
  Thr Pro Tyr Asp Asp Gln Gly His Gly Thr His Val Ala Gly Thr
          35                  40                  45
  GTT GCT GGT                                              145
  Val Ala Gly
```

(2) INFORMATION FOR SEQ ID NO:14
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 564
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (PCR fragment)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:
TCT CAC GGA ACT CAC GTG GCG GGA ACA GTT GCC GGA ACA GGC AGC 45

```
Ser His Gly Thr His Val Ala Gly Thr Val Ala Gly Thr Gly Ser
                  5                  10                  15
GTT AAC TCC CAG TAC ATA GGC GTC GCC CCC GGC GCG AAG CTC GTC      90
Val Asn Ser Gln Tyr Ile Gly Val Ala Pro Gly Ala Lys Leu Val
                 20                  25                  30
GGT GTC AAG GTT CTC GGT GCC GAC GGT TCG GGA AGC GTC TCC ACC     135
Gly Val Lys Val Leu Gly Ala Asp Gly Ser Gly Ser Val Ser Thr
                 35                  40                  45
ATC ATC GCG GGT GTT GAC TGG GTC GTC CAG AAC AAG GAT AAG TAC     180
Ile Ile Ala Gly Val Asp Trp Val Val Gln Asn Lys Asp Lys Tyr
                 50                  55                  60
GGG ATA AGG GTC ATC AAC CTC TCC CTC GGC TCC TCC CAG AGC TCC     225
Gly Ile Arg Val Ile Asn Leu Ser Leu Gly Ser Ser Gln Ser Ser
                 65                  70                  75
GAC GGA GCC GAC TCC CTC AGT CAG GCC GTC AAC AAC GCC TGG GAC     270
Asp Gly Ala Asp Ser Leu Ser Gln Ala Val Asn Asn Ala Trp Asp
                 80                  85                  90
GCC GGT ATA GTA GTC TGC GTC GCC GCC GGC AAC AGC GGG CCG AAC     315
Ala Gly Ile Val Val Cys Val Ala Ala Gly Asn Ser Gly Pro Asn
                 95                 100                 105
ACC TAC ACC GTC GGC TCA CCC GCC GCC GCG AGC AAG GTC ATA ACC     360
Thr Tyr Thr Val Gly Ser Pro Ala Ala Ala Ser Lys Val Ile Thr
                110                 115                 120
GTC GGT GCA GTT GAC AGC AAC GAC AAC ATC GCC AGC TTC TCC AGC     405
Val Gly Ala Val Asp Ser Asn Asp Asn Ile Ala Ser Phe Ser Ser
                125                 130                 135
AGG GGA CCG ACC GCG GAC GGA AGG CTC AAG CCG GAA GTC GTC GCC     450
Arg Gly Pro Thr Ala Asp Gly Arg Leu Lys Pro Glu Val Val Ala
                140                 145                 150
CCC GGC GTT GAC ATC ATA GCC CCG CGC GCC AGC GGA ACC AGC ATG     495
Pro Gly Val Asp Ile Ile Ala Pro Arg Ala Ser Gly Thr Ser Met
                155                 160                 165
GGC ACC CCG ATA AAC GAC TAC TAC ACC AAG GCC TCT GGA ACC TCA     540
Gly Thr Pro Ile Asn Asp Tyr Tyr Thr Lys Ala Ser Gly Thr Ser
                170                 175                 180
ATG GCC ACT CCC CAT GTT ACC GGT                                 564
Met Ala Thr Pro His Val Thr Gly
                185
```

(2) INFORMATION FOR SEQ ID NO:15
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 1859
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: genomic DNA

(vi) ORIGINAL SOURCE: Thermococcus celer
(B) STRAIN: DSM2476
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

```
GAGCTCCGAC GGAACCGACT CCCTCAGTCA GGCCGTCAAC AACGCCTGGG ACGCCGGTAT    60
AGTAGTCTGC GTCGCCGCCG GCAACAGCGG GCCGAACACC TACACCGTCG GCTCACCCGC   120
CGCCGCGAGC AAGGTCATAA CCGTCGGTGC AGTTGACAGC AACGACAACA TCGCCAGCTT   180
CTCCAGCAGG GGACCGACCG CGGACGGAAG GCTCAAGCCG GAAGTCGTCG CCCCCGGCGT   240
TGACATCATA GCCCCGCGCG CCAGCGGAAC CAGCATGGGC ACCCCGATAA ACGACTACTA   300
CACCAAGGCC TCTGGAACCA GCATGGCCAC CCCGCACGTT TCGGGCGTTG GCGCGCTCAT   360
CCTCCAGGCC CACCCGAGCT GGACCCCGGA CAAGGTGAAG ACCGCCCTCA TCGAGACCGC   420
CGACATAGTC GCCCCCAAGG AGATAGCGGA CATCGCCTAC GGTGCGGGTA GGGTGAACGT   480
CTACAAGGCC ATCAAGTACG ACGACTACGC CAAGCTCACC TTCACCGGCT CCGTCGCCGA   540
CAAGGGAAGC GCCACCCACA CCTTCGACGT CAGCGGCGCC ACCTTCGTGA CCGCCACCCT   600
CTACTGGGAC ACGGGCTCGA GCGACATCGA CCTCTACCTC TACGACCCCA ACGGGAACGA   660
GGTTGACTAC TCCTACACCG CCTACTACGG CTTCGAGAAG GTCGGCTACT ACAACCCGAC   720
CGCCGGAACC TGGACGGTCA AGGTCGTCAG CTACAAGGGC GCGGCGAACT ACCAGGTCGA   780
CGTCGTCAGC GACGGGAGCC TCAGCCAGTC CGGCGGCGGC AACCCGAATC CAAACCCCAA   840
CCCGAACCCA ACCCCGACCA CCGACACCCA GACCTTCACC GGTTCCGTTA ACGACTACTG   900
GGACACCAGC GACACCTTCA CCATGAACGT CAACAGCGGT GCCACCAAGA TAACCGGTGA   960
CCTGACCTTC GATACTTCCT ACAACGACCT CGACCTCTAC CTCTACGACC CCAACGGCAA  1020
CCTCGTTGAC AGGTCCACGT CGAGCAACAG CTACGAGCAC GTCGAGTACG CCAACCCCGC  1080
CCCGGGAACC TGGACGTTCC TCGTCTACGC CTACAGCACC TACGGCTGGG CGGACTACCA  1140
GCTCAAGGCC GTCGTCTACT ACGGGTGAAG GTTTTTAATC CCCTTTTCTT TCCCCTTTTG  1200
AGGTGGTTGG GATGAAGCGG GTTCTTGCGG CGATCCTTGT AATCATGCTC ATCGGATTAT  1260
CATTCCCTGC CGGAAGTGCT AAAATCGAGC CCTACGTTTA CAGCCCCACC GTTCCGGATA  1320
CCGCCTTCGC GGTTCTCACC CTGTACAGGA CCGGGGACTA CGCCCGGGTT CTCGAGGGAT  1380
ACGAGTGGCT CCTCCAGATG AGAACTCCCA TCGATTCGTG GGGGGTTTCC CGCGGGGAAA  1440
CGCACATGGC CAAGTACACG GCAATGGCGA TGCTGGCCCT CATGCGCGGC GAGAACGTGG  1500
CGAGGGGGCG TTACAGGGAT GTTCTCAACG ACGCCGCGTA CTGGTTAATA TACAAACAGA  1560
ACCCGGACGG CTCGTGGGAG GACTACACCG GAACGGCGCT GGCCGTCATC GCGCTCGGGG  1620
AGTTCCTTAA GGGCGGGTAC ATCAACGCGA ACCTGACCGG CTTCAAAAAG CAGGTTAAAG  1680
AGGCCGTAAA CCGCGGGGAA GGCTGGCTGA TGGATGCGGA CCCAAAAACG GACGCGGATA  1740
GAATATTCGG CTACCTCGCC CTCGGTAAAA AGGACGAACT CAAAAAGATG AACCCTTCCG  1800
GTGACCTGAA GGCCTACCGC GCCTTTGCAC TTGCCTACCT CGGGGAGAGG GTCGAGCTCG@ 1859
```

(2) INFORMATION FOR SEQ ID NO:16
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

```
TGTAGTAGTC GTTTATCGGG          20
```

(2) INFORMATION FOR SEQ ID NO:17

(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 1464
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: genomic DNA
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

```
AAGCTTAACA TCGAGCGCTC CACCTCTAAA GTAGGTGAGT GTGGATACGA AGGTTAGGGC   60
CGCTATGACG ACCTTCAGGA TCCCAACGGC TTCTTTTATG GGGAGCCCGG CGAAGGTGAG  120
AATTGAAAGG ATTACCATAC TCCCTCCGCT CATCATGGAG CCTATGAATC CCCCTCCAAA  180
AGAGAGAAGT GCTATAAGGA GCGTCCTCAT GTTCCATGCT ATGTTTTGGT ATTTAATGCT  240
TTTCCGCTTA ATGTTACACC TCCTCATGAC AATTTCGCGT TTAGGGATGG GGTTAATTGG  300
ACCCCTCCGA GCCACGGGTT GATGTCCATT ATGTCGATAT TCACCATCTT ATCCCCAACT  360
TTGTGGGTTT CAAACATTAC CCTACGTTAT ATTTTTATCG TCCTAATTAA CTGCTGAAAC  420
GGGCGCTTAT CGTTCATCGT TGATGGTTTT GGGTGACCGG GCATTAAGGA ATTGTGTCGT  480
TTGCTGAAAT TTATGAAACG GAGTTGGCTT CTTTATGTTA CATAAAGATG TACATTACTG  540
TAATGTATAT AAATGGAAGA AACACTGTTG CGTAAACTTT TTAATGTATC CAATATCAGT  600
ACTTCGATGT CCCGATATGG GACATGTTGG ATAGGAGGGT ACTGGAATGA AGAGGTTAGG  660
TGCTGTGGTG CTGGCACTGG TGCTCGTGGG TCTTCTGGCC GGAACGGCCC TTGCGGCACC  720
CGTAAAACCG GTTGTCAGGA ACAACGCGGT TCAGCAGAAG AACTACGGAC TGCTGACCCC  780
GGGACTGTTC AAGAAAGTCC AGAGGATGAA CTGGAACCAG GAAGTGGACA CCGTCATAAT  840
GTTCGGGAGC TACGGAGACA GGGACAGGGC GGTTAAGGTA CTGAGGCTCA TGGGCGCCCA  900
GGTCAAGTAC TCCTACAAGA TAATCCCTGC TGTCGCGGTT AAAATAAAGG CCAGGGACCT  960
TCTGCTGATC GCGGGCATGA TAGACACGGG TTACTTCGGT AACACAAGGG TCTCGGGCAT 1020
AAAGTTCATA CAGGAGGATT ACAAGGTTCA GGTTGACGAC GCCACTTCCG TCTCCCAGAT 1080
AGGGGCCGAT ACCGTCTGGA ACTCCCTCGG CTACGACGGA AGCGGTGTGG TGGTTGCCAT 1140
CGTCGATACG GGTATAGACG CGAACCACCC CGATCTGAAG GGCAAGGTCA TAGGCTGGTA 1200
CGACTCCGTC AACGGCAGGT CGACCCCCTA CGATGACCAG GGACACGGAA CCCACGTTGC 1260
GGGTATCGTT GCCGGAACCG GGAGCGTTAA CTCCCAGTAC ATAGGCGTCG GCCCCGGCGC 1320
GAAGCTCGTC GGCGTCAAGG TTCTCGGTTC CGACGGTTCG GGAAGCGTCT CCACCATCAT 1380
CGCGGGTGTT GACTGGAACG TCCAGAACTA GGACAAGTAC GGGATAAGGG TCATCAACCT 1440
CTCCCTCGGC TCCTCCCAGA GCTC                                        1464
```

(2) INFORMATION FOR SEQ ID NO:18
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 33
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 18:

```
AAAAGAATTC GGATCCATGA AGAGGTTAGG TGC                33
```

(2) INFORMATION FOR SEQ ID NO:19
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 28

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 19:
TTTTATCGAT CAGGCGTCCC AGGCGTTG 28

(2) INFORMATION FOR SEQ ID NO:20
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 20:
CATTATAGGT AAGAGAGGAA TG 22

(2) INFORMATION FOR SEQ ID NO:21
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 30
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 21:
GATCCATTCC TCTCTTACCT ATAATGGTAC 30

(2) INFORMATION FOR SEQ ID NO:22
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 22:
TAGCAGTAAT TGACACGGG 19

(2) INFORMATION FOR SEQ ID NO:23
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 19
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 23:
TAGCAGTAAT TGACACTGG 19

(2) INFORMATION FOR SEQ ID NO:24
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 24:
CTGTTCCAGC TACGTGAGTT CC 22

(2) INFORMATION FOR SEQ ID NO:25
(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 25:
CTGTTCCAGC TACATGAGTT CC 22

(2) INFORMATION FOR SEQ ID NO:26
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 507
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: genomic DNA
(vi) ORIGINAL SOURCE: Pyrococcus furiosus
(B) STRAIN: DSM3638
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 26:

```
A CTA GTC ATC TCA GGT TTA ACA GGG GGT AAA GCT AAG CTT TCA GGT      46
  Leu Val Ile Ser Gly Leu Thr Gly Gly Lys Ala Lys Leu Ser Gly
    5                  10                  15
  GTT AGG TTT ATC CAG GAA GAC TAC AAA GTT ACA GTT TCA GCA GAA      91
  Val Arg Phe Ile Gln Glu Asp Tyr Lys Val Thr Val Ser Ala Glu
              20                  25                  30
  TTA GAA GGA CTG GAT GAG TCT GCA GCT CAA GTT ATG GCA ACT TAC     136
  Leu Glu Gly Leu Asp Glu Ser Ala Ala Gln Val Met Ala Thr Tyr
              35                  40                  45
  GTT TGG AAC TTG GGA TAT GAT GGT TCT GGA ATC ACA ATA GGA ATA     181
  Val Trp Asn Leu Gly Tyr Asp Gly Ser Gly Ile Thr Ile Gly Ile      60
              50                  55                  60
  ATT GAC ACT GGA ATT GAC GCT TCT CAT CCA GAT CTC CAA GGA AAA     226
  Ile Asp Thr Gly Ile Asp Ala Ser His Pro Asp Leu Gln Gly Lys      75
              65                  70                  75
```

```
GTA ATT GGG TGG GTA GAT TTT GTC AAT GGT AGG AGT TAT CCA TAC      271
Val Ile Gly Trp Val Asp Phe Val Asn Gly Arg Ser Tyr Pro Tyr      90
                80                  85                  90
GAT GAC CAT GGA CAT GGA ACT CAT GTA GCT TCA ATA GCA GCT GGT      316
Asp Asp His Gly His Gly Thr His Val Ala Ser Ile Ala Ala Gly      105
                95                  100                 105
ACT GGA GCA GCA AGT AAT GGC AAG TAC AAG GGA ATG GCT CCA GGA      361
Thr Gly Ala Ala Ser Asn Gly Lys Tyr Lys Gly Met Ala Pro Gly      120
                110                 115                 120
GCT AAG CTG GCG GGA ATT AAG GTT CTA GGT GCC GAT GGT TCT GGA      406
Ala Lys Leu Ala Gly Ile Lys Val Leu Gly Ala Asp Gly Ser Gly      135
                125                 130                 135
AGC ATA TCT ACT ATA ATT AAG GGA GTT GAG TGG GCC GTT GAT AAC      451
Ser Ile Ser Thr Ile Ile Lys Gly Val Glu Trp Ala Val Asp Asn      150
                140                 145                 150
AAA GAT AAG TAC GGA ATT AAG GTC ATT AAT CTT TCT CTT GGT TCA      496
Lys Asp Lys Tyr Gly Ile Lys Val Ile Asn Leu Ser Leu Gly Ser      165
                155                 160                 165
AGC CAG AGC TC                                                  507
Ser Gln Ser                                                     168
```

(2) INFORMATION FOR SEQ ID NO:27
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 30
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 27:
TGACACTGGA ATTGACGCTT CTCATCCAGA 30

(2) INFORMATION FOR SEQ ID NO:28
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 30
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 28:
TCTCCAAGGA AAAGTAATTG GGTGGGTAGA 30

(2) INFORMATION FOR SEQ ID NO:29
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 30
(B) TYPE: nucleic acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 29:

```
GTTGCCATAA CTTGAGCTGC AGACTCATCC                30
```

(2) INFORMATION FOR SEQ ID NO:30
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 420
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (PCR fragment)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 30:

```
TTTATTAAGC ATAAAATAGC CATGCAACTT TGATCACTAA TGTGCGGTGG TGCAC ATG    59
                                                            Met
   AAG GGG CTG AAA GCT CTC ATA TTA GTG ATT TTA GTT CTA GGT TTG      104
   Lys Gly Leu Lys Ala Leu Ile Leu Val Ile Leu Val Leu Gly Leu
     5                  10                  15
   GTA GTA GGG AGC GTA GCG GCA GCT CCA GAG AAG AAA GTT GTT CAA      149
   Val Val Gly Ser Val Ala Ala Ala Pro Glu Lys Lys Val Val Gln
                 20                  25                  30
   GTA AGA AAT GTT GAG AAG AAC TAT GGT CTG CTA ACG CCA GGA CTG      194
   Val Arg Asn Val Glu Lys Asn Tyr Gly Leu Leu Thr Pro Gly Leu
                 35                  40                  45
   TTC AGA AAA ATT CCC AAA TTG GAT CCT AAC GAG GGA ATC AGC ACA      239
   Phe Arg Lys Ile Pro Lys Leu Asp Pro Asn Glu Gly Ile Ser Thr
                 50                  55                  60
   GTA ATT GTA TTT GTT AAC CAT AGG GGA AAA GAA ATT GCA GTA AGA      284
   Val Ile Val Phe Val Asn His Arg Gly Lys Glu Ile Ala Val Arg
                 65                  70                  75
   GTT CTT GAG TTA ATG GGT GCC CAA GTT AGG TAT GTG TAC CAT ATT      329
   Val Leu Glu Leu Met Gly Ala Gln Val Arg Tyr Val Tyr His Ile
                 80                  85                  90
   ATA CCC CCA ATA GCT GCC GAT CTT AAG GTT AGA GAC TTA CTA GTC      374
   Ile Pro Pro Ile Ala Ala Asp Leu Lys Val Arg Asp Leu Leu Val
                 95                 100                 105
   ATC TCA GGT TTA ACA GGG GGT GAA ACT AAG CTT TCA GGT GTT AGG T    420
   Ile Ser Gly Leu Thr Gly Gly Glu Thr Lys Leu Ser Gly Val Arg
                110                 115                 120
```

(2) INFORMATION FOR SEQ ID NO:31
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 180
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double

(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (PCR fragment)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 31:

GCTCTAGACT CTGGGAGGAG TAGTTATACT TGATGAAGCC TATTCTGAGT TTTCGGGAAA 60
AAGCTTCATA CCAAAAATCA GTGAGTATGA AAATTTAGTA ATTCTAAGGA CGTTTTCAAA 120
GGCGTTTGGA CTTGCTGGAA TTAGATGTGG ATATATGATA GCAAATGAAA AGATTATAGA 180

(2) INFORMATION FOR SEQ ID NO:32
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 28
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 32:

AGAGGGATCC ATGAAGGGGC TGAAAGCT 28

(2) INFORMATION FOR SEQ ID NO:33
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 28
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 33:

AGAGGCATGC GCTCTAGACT CTGGGAGAGT 28

(2) INFORMATION FOR SEQ ID NO:34
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 1962
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: genomic DNA
(vi) ORIGINAL SOURCE: Pyrococcus furiosus
(B) STRAIN: DSM3638
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 34:

ATGAAGGGGC TGAAAGCTCT CATATTAGTG ATTTTAGTTC TAGGTTTGGT AGTAGGGAGC 60
GTAGCGGCAG CTCCAGAGAA GAAAGTTGAA CAAGTAAGAA ATGTTGAGAA GAACTATGGT 120
CTGCTAACGC CAGGACTGTT CAGAAAAATT CAAAAATTGA ATCCTAACGA GGAAATCAGC 180
ACAGTAATTG TATTTGAAAA CCATAGGGAA AAAGAAATTG CAGTAAGAGT TCTTGAGTTA 240
ATGGGTGCAA AAGTTAGGTA TGTGTACCAT ATTATACCCG CAATAGCTGC CGATCTTAAG 300
GTTAGAGACT TACTAGTCAT CTCAGGTTTA ACAGGGGGTA AAGCTAAGCT TTCAGGTGTT 360
AGGTTTATCC AGGAAGACTA CAAAGTTACA GTTTCAGCAG AATTAGAAGG ACTGGATGAG 420
TCTGCAGCTC AAGTTATGGC AACTTACGTT TGGAACTTGG GATATGATGG TTCTGGAATC 480
ACAATAGGAA TAATTGACAC TGGAATTGAC GCTTCTCATC CAGATCTCCA AGGAAAAGTA 540

```
ATTGGGTGGG TAGATTTTGT CAATGGTAGG AGTTATCCAT ACGATGACCA TGGACATGGA   600
ACTCATGTAG CTTCAATAGC AGCTGGTACT GGAGCAGCAA GTAATGGCAA GTACAAGGGA   660
ATGGCTCCAG GAGCTAAGCT GGCGGGAATT AAGGTTCTAG GTGCCGATGG TTCTGGAAGC   720
ATATCTACTA TAATTAAGGG AGTTGAGTGG GCCGTTGATA ACAAAGATAA GTACGGAATT   780
AAGGTCATTA ATCTTTCTCT TGGTTCAAGC CAGAGCTCAG ATGGTACTGA CGCTCTAAGT   840
CAGGCTGTTA ATGCAGCGTG GGATGCTGGA TTAGTTGTTG TGGTTGCCGC TGGAAACAGT   900
GGACCTAACA AGTATACAAT CGGTTCTCCA GCAGCTGCAA GCAAAGTTAT TACAGTTGGA   960
GCCGTTGACA AGTATGATGT TATAACAAGC TTCTCAAGCA GAGGGCCAAC TGCAGACGGC  1020
AGGCTTAAGC CTGAGGTTGT TGCTCCAGGA AACTGGATAA TTGCTGCCAG AGCAAGTGGA  1080
ACTAGCATGG GTCAACCAAT TAATGACTAT TACACAGCAG CTCCTGGGAC ATCAATGGCA  1140
ACTCCTCACG TAGCTGGTAT TGCAGCCCTC TTGCTCCAAG CACACCCGAG CTGGACTCCA  1200
GACAAAGTAA AAACAGCCCT CATAGAAACT GCTGATATCG TAAAGCCAGA TGAAATAGCC  1260
GATATAGCCT ACGGTGCAGG TAGGGTTAAT GCATACAAGG CTATAAACTA CGATAACTAT  1320
GCAAAGCTAG TGTTCACTGG ATATGTTGCC AACAAAGGCA GCCAAACTCA CCAGTTCGTT  1380
ATTAGCGGAG CTTCGTTCGT AACTGCCACA TTATACTGGG ACAATGCCAA TAGCGACCTT  1440
GATCTTTACC TCTACGATCC CAATGGAAAC CAGGTTGACT ACTCTTACAC CGCCTACTAT  1500
GGATTCGAAA AGGTTGGTTA TTACAACCCA ACTGATGGAA CATGGACAAT TAAGGTTGTA  1560
AGCTACAGCG GAAGTGCAAA CTATCAAGTA GATGTGGTAA GTGATGGTTC CCTTTCACAG  1620
CCTGGAAGTT CACCATCTCC ACAACCAGAA CCAACAGTAG ACGCAAAGAC GTTCCAAGGA  1680
TCCGATCACT ACTACTATGA CAGGAGCGAC ACCTTTACAA TGACCGTTAA CTCTGGGGCT  1740
ACAAAGATTA CTGGAGACCT AGTGTTTGAC ACAAGCTACC ATGATCTTGA CCTTTACCTC  1800
TACGATCCTA ACCAGAAGCT TGTAGATAGA TCGGAGAGTC CCAACAGCTA CGAACACGTA  1860
GAATACTTAA CCCCCGCCCC AGGAACCTGG TACTTCCTAG TATATGCCTA CTACACTTAC  1920
GGTTGGGCTT ACTACGAGCT GACGGCTAAA GTTTATTATG GC                      1962
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(2) INFORMATION FOR SEQ ID NO:35
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 654
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 35:

```
Met Lys Gly Leu Lys Ala Leu Ile Leu Val Ile Leu Val Leu Gly
  5                  10                  15
Leu Val Val Gly Ser Val Ala Ala Ala Pro Glu Lys Lys Val Glu
            20                  25                  30
Gln Val Arg Asn Val Glu Lys Asn Tyr Gly Leu Leu Thr Pro Gly
        35                  40                  45
Leu Phe Arg Lys Ile Gln Lys Leu Asn Pro Asn Glu Glu Ile Ser
    50                  55                  60
Thr Val Ile Val Phe Glu Asn His Arg Glu Lys Glu Ile Ala Val
65                  70                  75
Arg Val Leu Glu Leu Met Gly Ala Lys Val Arg Tyr Val Tyr His
                80                  85                  90
Ile Ile Pro Ala Ile Ala Ala Asp Leu Lys Val Arg Asp Leu Leu
```

95 100 105
Val Ile Ser Gly Leu Thr Gly Gly Lys Ala Lys Leu Ser Gly Val
110 115 120
Arg Phe Ile Gln Glu Asp Tyr Lys Val Thr Val Ser Ala Glu Leu
125 130 135
Glu Gly Leu Asp Glu Ser Ala Ala Gln Val Met Ala Thr Tyr Val
140 145 150
Trp Asn Leu Gly Tyr Asp Gly Ser Gly Ile Thr Ile Gly Ile Ile
155 160 165
Asp Thr Gly Ile Asp Ala Ser His Pro Asp Leu Gln Gly Lys Val
170 175 180
Ile Gly Trp Val Asp Phe Val Asn Gly Arg Ser Tyr Pro Tyr Asp
185 190 195
Asp His Gly His Gly Thr His Val Ala Ser Ile Ala Ala Gly Thr
200 205 210
Gly Ala Ala Ser Asn Gly Lys Tyr Lys Gly Met Ala Pro Gly Ala
215 220 225
Lys Leu Ala Gly Ile Lys Val Leu Gly Ala Asp Gly Ser Gly Ser
230 235 240
Ile Ser Thr Ile Ile Lys Gly Val Glu Trp Ala Val Asp Asn Lys
245 250 255
Asp Lys Tyr Gly Ile Lys Val Ile Asn Leu Ser Leu Gly Ser Ser
260 265 270
Gln Ser Ser Asp Gly Thr Asp Ala Leu Ser Gln Ala Val Asn Ala
275 280 285
Ala Trp Asp Ala Gly Leu Val Val Val Val Ala Ala Gly Asn Ser
290 295 300
Gly Pro Asn Lys Tyr Thr Ile Gly Ser Pro Ala Ala Ala Ser Lys
305 310 315
Val Ile Thr Val Gly Ala Val Asp Lys Tyr Asp Val Ile Thr Ser
320 325 330
Phe Ser Ser Arg Gly Pro Thr Ala Asp Gly Arg Leu Lys Pro Glu
335 340 345
Val Val Ala Pro Gly Asn Trp Ile Ile Ala Ala Arg Ala Ser Gly
350 355 360
Thr Ser Met Gly Gln Pro Ile Asn Asp Tyr Tyr Thr Ala Ala Pro
365 370 375
Gly Thr Ser Met Ala Thr Pro His Val Ala Gly Ile Ala Ala Leu
380 385 390
Leu Leu Gln Ala His Pro Ser Trp Thr Pro Asp Lys Val Lys Thr
395 400 405
Ala Leu Ile Glu Thr Ala Asp Ile Val Lys Pro Asp Glu Ile Ala
410 415 420
Asp Ile Ala Tyr Gly Ala Gly Arg Val Asn Ala Tyr Lys Ala Ile
425 430 435
Asn Tyr Asp Asn Tyr Ala Lys Leu Val Phe Thr Gly Tyr Val Ala

```
                    440                 445                 450
Asn Lys Gly Ser Gln Thr His Gln Phe Val Ile Ser Gly Ala Ser
                    455                 460                 465
Phe Val Thr Ala Thr Leu Tyr Trp Asp Asn Ala Asn Ser Asp Leu
                    470                 475                 480
Asp Leu Tyr Leu Tyr Asp Pro Asn Gly Asn Gln Val Asp Tyr Ser
                    485                 490                 495
Tyr Thr Ala Tyr Tyr Gly Phe Glu Lys Val Gly Tyr Tyr Asn Pro
                    500                 505                 510
Thr Asp Gly Thr Trp Thr Ile Lys Val Val Ser Tyr Ser Gly Ser
                    515                 520                 525
Ala Asn Tyr Gln Val Asp Val Val Ser Asp Gly Ser Leu Ser Gln
                    530                 535                 540
Pro Gly Ser Ser Pro Ser Pro Gln Pro Glu Pro Thr Val Asp Ala
                    545                 550                 555
Lys Thr Phe Gln Gly Ser Asp His Tyr Tyr Tyr Asp Arg Ser Asp
                    560                 565                 570
Thr Phe Thr Met Thr Val Asn Ser Gly Ala Thr Lys Ile Thr Gly
                    575                 580                 585
Asp Leu Val Phe Asp Thr Ser Tyr His Asp Leu Asp Leu Tyr Leu
                    590                 595                 600
Tyr Asp Pro Asn Gln Lys Leu Val Asp Arg Ser Glu Ser Pro Asn
                    605                 610                 615
Ser Tyr Glu His Val Glu Tyr Leu Thr Pro Ala Pro Gly Thr Trp
                    620                 625                 630
Tyr Phe Leu Val Tyr Ala Tyr Tyr Thr Tyr Gly Trp Ala Tyr Tyr
                    635                 640                 645
Glu Leu Thr Ala Lys Val Tyr Tyr Gly
                    650
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(2) INFORMATION FOR SEQ ID NO:36
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 25
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 36:
TCTGAATTCG TTCTTTTCTG TATGG 25

(2) INFORMATION FOR SEQ ID NO:37
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 37:
TGTACTGCTG GATCCGGCAG 20

(2) INFORMATION FOR SEQ ID NO:38
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 80
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: genomic DNA
(vi) ORIGINAL SOURCE: Pyrococcus furiosus
(B) STRAIN: DSM3638
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 38:
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ATATCTCCCG CGGGTAAGGT 80

(2) INFORMATION FOR SEQ ID NO:39
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 30
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 39:
AGAGGCATGC GTATCCATCA GATTTTTGAG 30

(2) INFORMATION FOR SEQ ID NO:40
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 40:
AGTGAACGGA TACTTGGAAC 20

(2) INFORMATION FOR SEQ ID NO:41
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 20
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: other nucleic acid (synthetic DNA)
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 41:
GTTCCAAGTA TCCGTTCACT 20

(2) INFORMATION FOR SEQ ID NO:42
(i) SEQUENCE CHARACTERISTICS
(A) LENGTH: 12
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear
(ii) MOLECULE TYPE: peptide
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 42:

Ala Glu Leu Glu Gly Leu Asp Glu Ser Ala Ala Gln
5 10

Claims

1. A hyperthermostable protease having an amino acid sequence represented by SEQ ID No. 1 of the Sequence Listing or functional equivalents thereof.

2. A hyperthermostable protease gene encoding the hyperthermostable protease or functional equivalents thereof as defined in claim 1.

3. The hyperthermostable protease gene according to claim 2, which has a nucleotide sequence represented by SEQ ID No. 2 of the Sequence Listing.

4. The hyperthermostable protease gene according to claim 2, which hybridizes to the hyperthermostable protease gene as defined in claim 3.

5. A hyperthermostable protease having an amino acid sequence represented by SEQ ID No. 3 of the Sequence Listing or functional equivalents thereof.

6. A hyperthermostable protease gene encoding the hyperthermostable protease or functional equivalents thereof as defined in claim 5.

7. The hyperthermostable protease gene according to claim 6, which has an nucleotide sequence represented by SEQ ID No. 4 of the Sequence Listing.

8. The hyperthermostable protease gene according to claim 6, which hybridizes to the hyperthermostable protease gene as defined in claim 7.

9. A hyperthermostable protease having an amino acid sequence represented by SEQ ID No. 5 of the Sequence Listing or functional equivalents thereof.

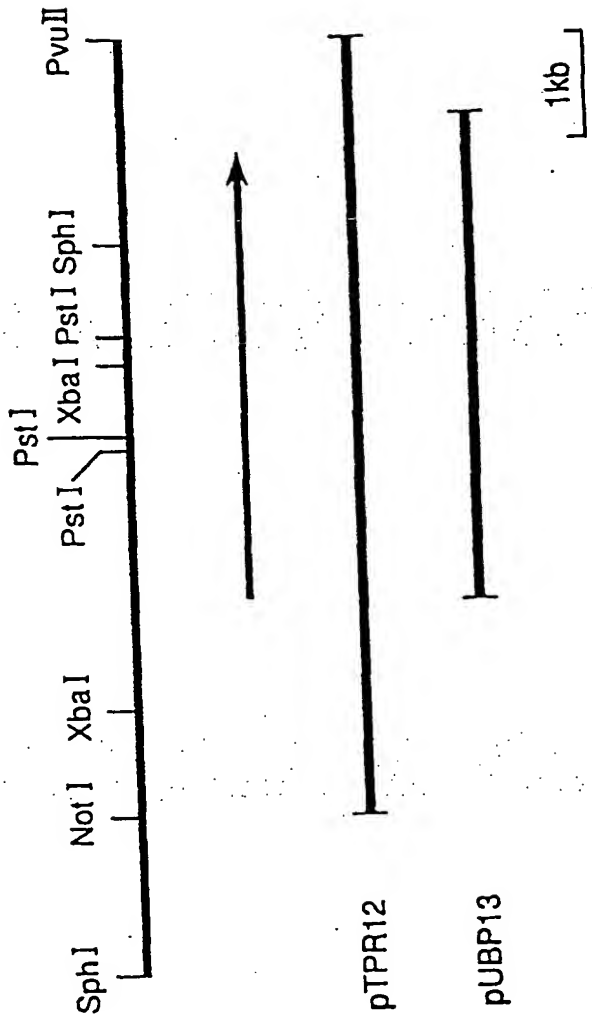
10. A hyperthermostable protease gene encoding the hyperthermostable protease or functional equivalents thereof as defined in claim 9.

11. The hyperthermostable protease gene according to claim 10, which has a nucleotide sequence represented by SEQ ID No. 6 of the Sequence Listing.

12. The hyperthermostable protease gene according to claim 10, which hybridizes to the hyperthermostable protease gene according to claim 11.

13. A method for preparing a hyperthermostable protease which comprises culturing a transformant containing any one of hyperthermostable protease genes as defined in claims 2-4, 6-8 and 10-12, then harvesting a hyperthermostable protease from the culture.

Fig. 1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Not I
Xba I
Pst I
Pst I
Xba I
Pst I
Sph I
Pvu II
pTPR12
pUBP13
1kb

Fig. 2

```
                170                 175                 180
          Asp Gly Ser Gly Val Val Val Ala Val Leu Asp Thr Gly Val
5'-GAT GGT AGT GGT GTT GTT GTT GCA GTA CTT GAC ACG GGA GTT-3'

PRO-1F    5'-GGW WSD RRT GTT RRH GTH GCD GTD MTY GAC ACB GG-3'
```

Fig. 3

```
                365                 370                 375
          His Gly His Gly Thr His Val Ala Gly Thr Val Ala Gly Tyr
5'-CAC GGT CAC GGA ACT CAC GTA GCT GGA ACT GTT GCT GGT TAC-3'

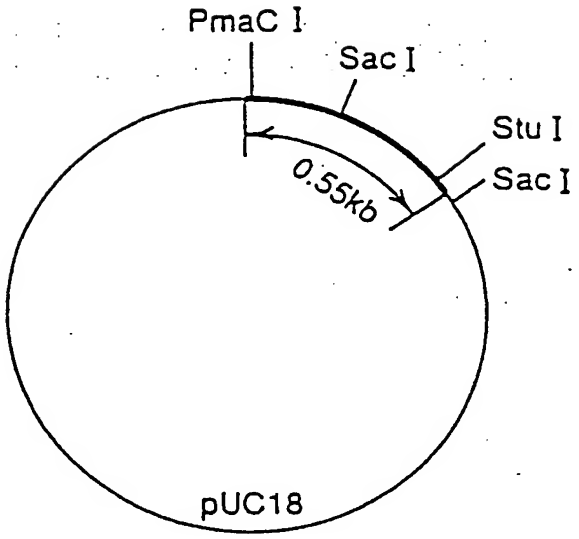
PRO-2F    5'-KST CAC GGA ACT CAC GTD GCB GGH ACD GTT GC-3'
PRO-2R         3'-GTG CCT TGA GTG CAH CGV CCK TGH CAA CGM CSA-5'
```

Fig. 4

```
                590                 595
          Ser Gly Thr Ser Met Ala Thr Pro His Val Ser Gly Val Val
5'-TCT GGA ACT TCG ATG GCT ACT CCA CAT GTC AGC GGT GTC GTT-3'

PRO-4R    3'-CCD TGV AGB TAC CGD WGA GGB GTR CAV YSG CCH C-5'
```

Fig. 5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Stu I
Sac I
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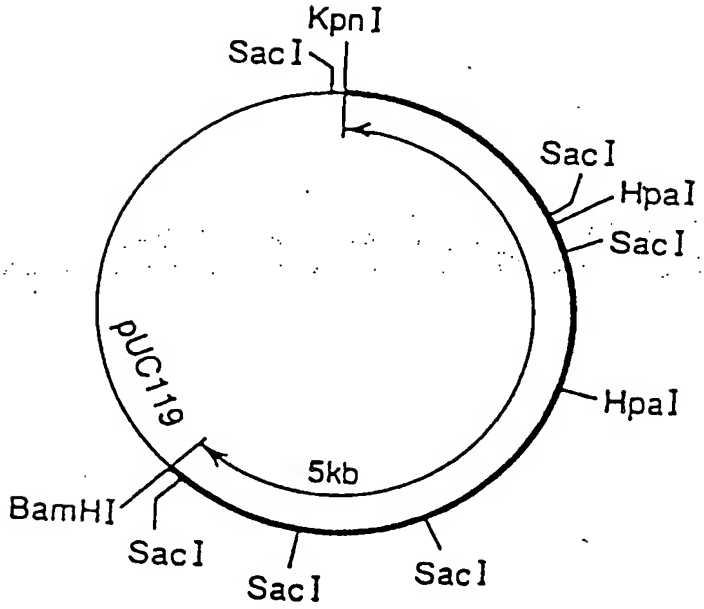
pUC18

Fig. 6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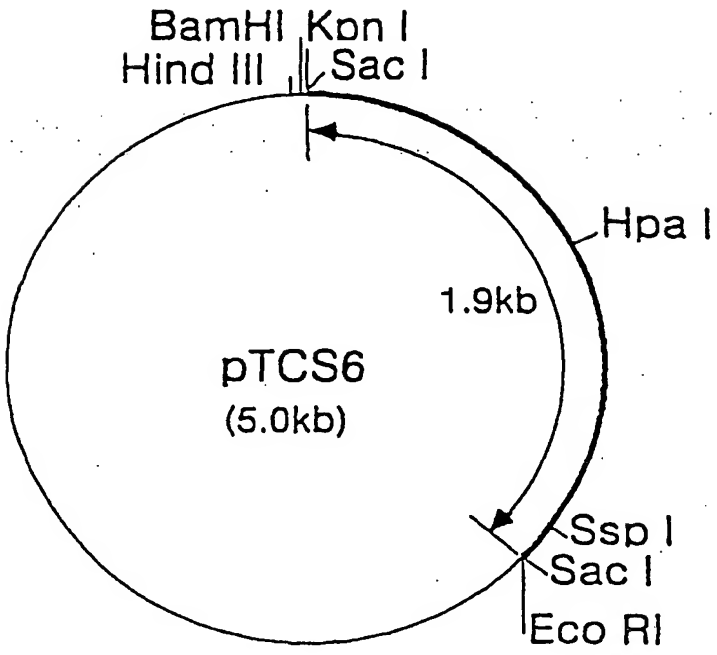
BamHI
Sac I
Sac I
Sac I

Fig. 7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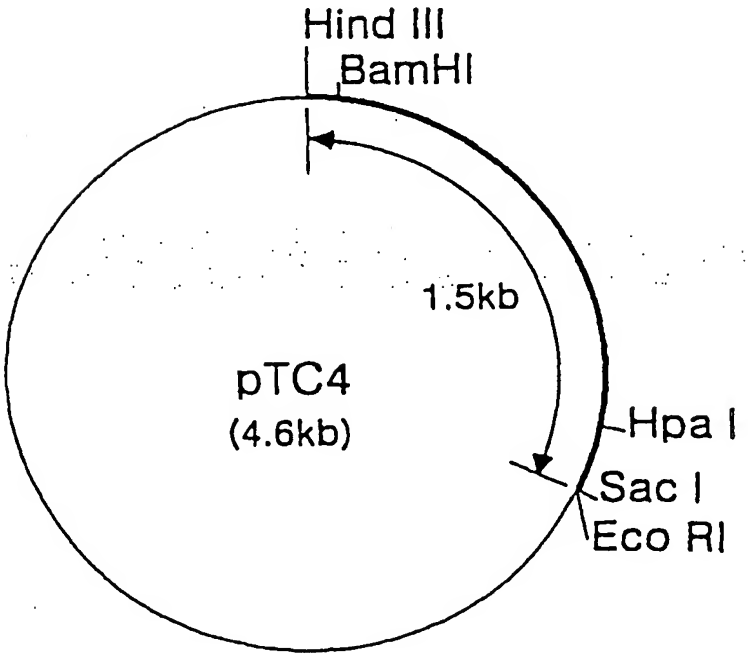
Ssp I
Sac I
Eco RI

Fig. 8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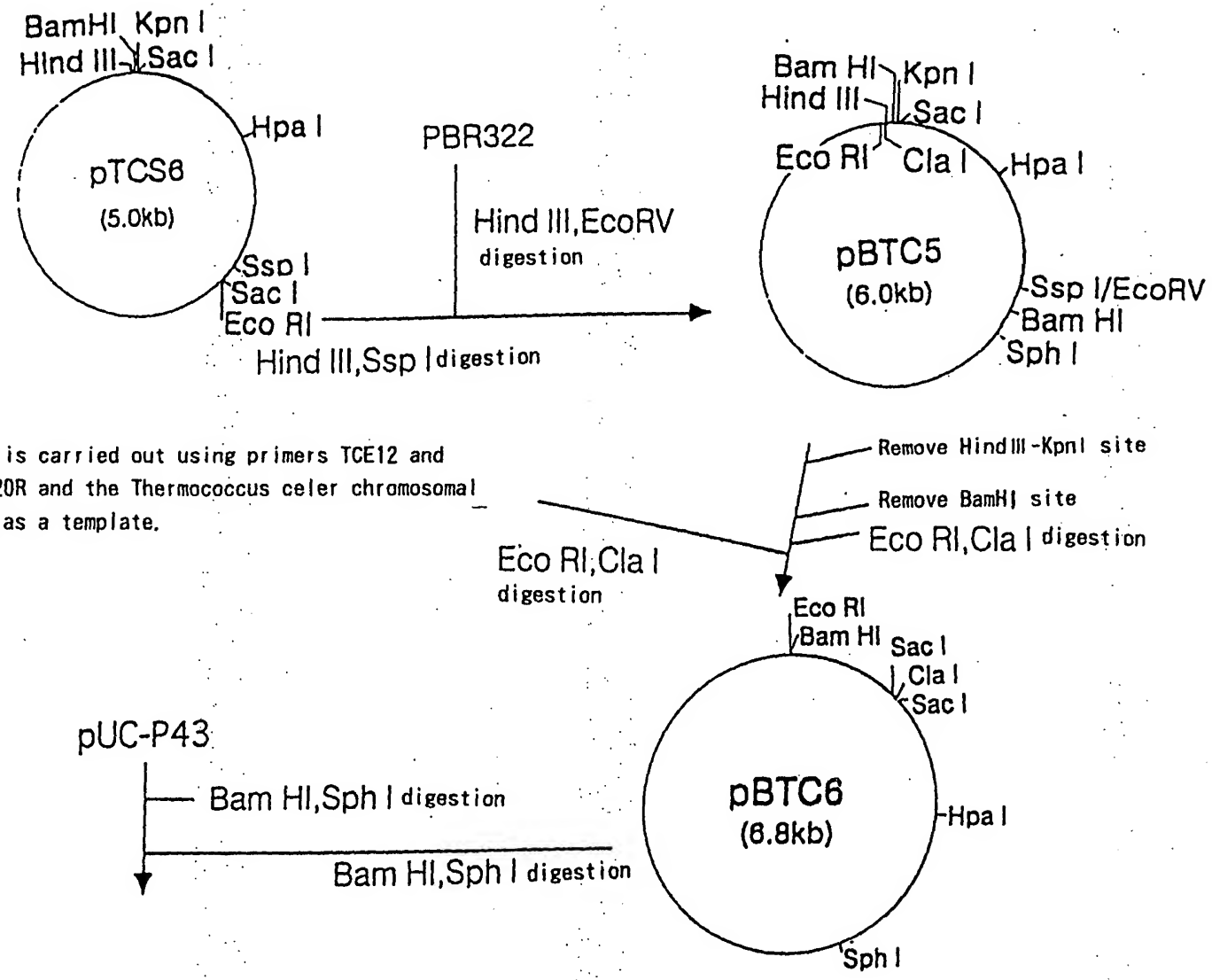
Hpa I
Sac I
Eco RI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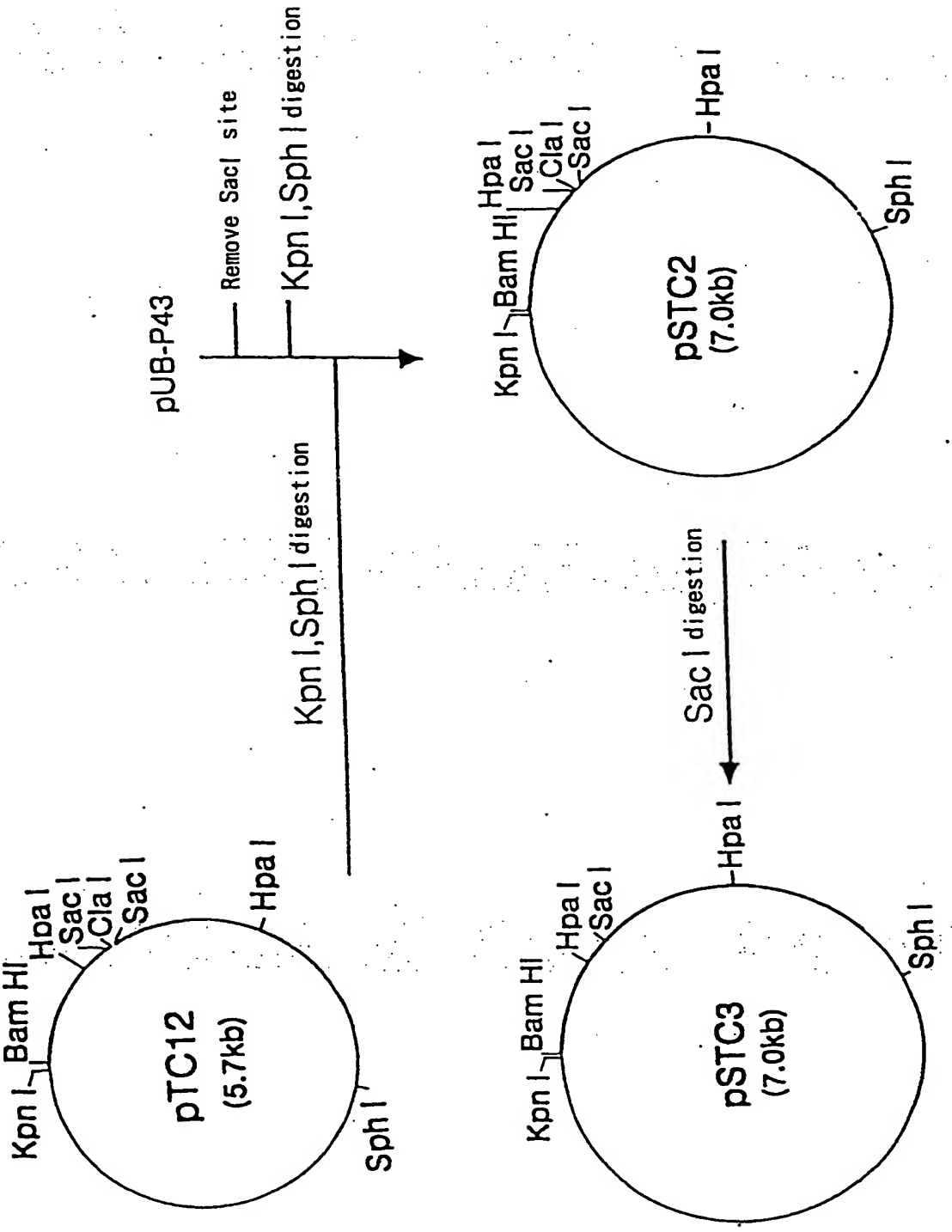
Hind III Sac I
Hpa I
pTCS6
(5.0kb)
Ssp I
Sac I
Eco RI
PBR322
Hind III,EcoRV
digestion
Hind III,Ssp I digestion
Bam HI Kpn I
Hind III Sac I
Eco RI Cla I
Hpa I
pBTC5
(6.0kb)
Ssp I/EcoRV
Bam HI
Sph I
PCR is carried out using primers TCE12 and TCE20R and the Thermococcus celer chromosomal DNA as a template.
Remove HindIII-KpnI site
Remove BamHI site
Eco RI,Cla I digestion
Eco RI,Cla I
digestion
Eco RI
Bam HI
Sac I
Cla I
Sac I
pUC-P43
Bam HI,Sph I digestion
Bam HI,Sph I digestion
pBTC6
(6.8kb)
Hpa I
Sph I

Fig. 9

Fig. 9 (Cont'd)


pUB-P43
Remove SacI site
Kpn I,Sph I digestion
Kpn I,Sph I digestion
Sac I digestion
pTC12
(5.7kb)
Kpn I
Bam HI
Hpa I
Sac I
Cla I
Sac I
Hpa I
Sph I
pSTC2
(7.0kb)
Kpn I
Bam HI
Hpa I
Sac I
Cla I
Sac I
Hpa I
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Kpn I
Bam HI
Hpa I
Sac I
Hpa I
Sph I

Fig. 10

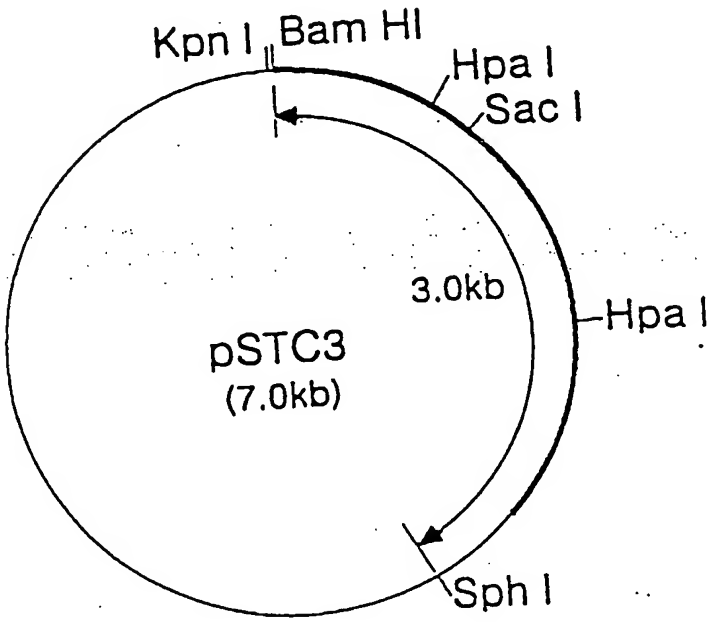


Fig. 11

```
                10        20        30        40        50
PFUL    MNKKGLTVLF IAIMLLSVVP VHFVSAETPP VSSENSTTSI LPNQQVVTKE
TCES                MKRLGAVV LALVLVGLLA GTALAAPVKP VVRNNAVQQK
SUBTILISIN                                        MRGKKVWISL

                60        70        80        90       100
PFUL    VSQAALNAIM KGQPNMVLII KTKEGKLEEA KTELEKLGAE ILDENRVLNM
TCES    NYGLLTPGLF KKVQRMNWNQ EVDTVIMFGS YGDRDRAVKV LRLMGAQVKY
SUBTILISIN LFALALIFTM AFGSTSSAQA AGKSNGEKKY IVGFKQTMST MSAAKKKDVI

               110       120       130       140       150
PFUL    LLVKIKPEKV KELNYISSLE KAWLNREVKL SPPIVEKDVK TKEPSLEPKM
TCES    SYKIIPAVAV KIKARDLLLI AGMIDTGYFG NTRVSGIKFI QEDYKVQVDD
SUBTILISIN SEKGGKVQKQ FKYVDAASAT LNEKAVKELK KDPSVAYVEE DHVAHAYAQS

               160       170       180       190       200
PFUL    YNSTWVINAL QFIQEFGYDG SGVVVAVLDT GVDPNHEFLS ITPDGRKII
TCES    ATSVSQIGAD TVWNSLGYDG SGVVVAIVDT GIDANHPDLK GKVIGWYDAV
SUBTILISIN VPYGVSQIKA PALHSQGYTG SNVKVAVIDS GIDSSHPDLK VAGGASMVPS

               210       220       230       240       250
PFUL    EWKDFTDEGF VDTSFSFSKV VNGTLIINTT FQVASGLTLN ESTGLMEYVV
TCES    NGRSTPYDDQ ---------- ---------- ---------- ----------
SUBTILISIN ETNPFQDNN- ---------- ---------- ---------- ----------

               260       270       280       290       300
PFUL    KTVYVSNVTI GNITSANGIY HFGLLPERYF DLNFDGDQED FYPVLLVNST
TCES    ---------- ---------- ---------- ---------- ----------
SUBTILISIN ---------- ---------- ---------- ---------- ----------
```

Fig. 11 (Cont'd)

```
                310       320       330       340       350
PFUL    GNGYDIAYVD TDLDYDFTDE VPLGQYNVTY DVAVFSYYYG PLNYVLAEID
TCES    ---------- ---------- ---------- ---------- ----------
SUBTILISIN ------- ---------- ---------- ---------- ----------

                360       370       380       390       400
PFUL    PNGEYAVFGW DGHGHGTHVA GTVAGVDSNT DAWDWLSMYS GEWEVFSRLY
TCES    ---------- ---GHGTHVA GIVAGTGSVN SQ--------- ----------
SUBTILISIN ------- ---SHGTHVA GTVAA--LNN SI--------- ----------

                410       420       430       440       450
PFUL    GWDYTNVTTD TVQGVAPGAQ IMAIRVLRSD GRGSMWDIIE CMTYAATHGA
TCES    ---------- -YIGVAPGAK LVGVKVLGAD GSGSVSTIIA CVDWVVQNKD
SUBTILISIN ------- GVLGVAPSAS LYAVKVLGAD GSGQYSWIIN GIEWAIANNM

                460       470       480       490       500
PFUL    ----DVISMS LGGNAPYLDG TDPESVAVDE LTEKYGVVFV IAAGNEGPGI
TCES    KYGIRVINLS LGSSQSS-DG TDSISQAVNN AWDA-GIVVC VAAGNSGPNT
SUBTILISIN ----DVINMS LGGP----SG SAALKAAVDK AVAS-GVVVV AAAGNEGTSG

                510       520       530       540       550
PFUL    N--IVGSPGV ATKAITVGAA AVPINVGVYV SQALGYPDYY GFYYFPAYTN
TCES    Y--TVGSPAA ASKVITVGAV DSNDN----- ---------- ----------
SUBTILISIN SSSTVGYPGK YPSVIAVGAV DSSNQ----- ---------- ----------

                560       570       580       590       600
PFUL    VRIAFSSRGP RIDGEIKPDV VAPGVGIYSS SLPMWIGGAD F---------MS
TCES    --IASFSSRG PTADGRLKPE VVAPGVDIIA PRASGTSMGT PINDYYTKAS
SUBTILISIN --RASFSSVG PELD------ VMAPGVSIQS TLPGNKYGA- ---------YN
```

Fig. 12

```
                610       620       630       640       650
PFUL      GTSMATPHVS GVVALLISGA KAEGIYYNPD IIKKVLESGA TWLEGDPYTG
TCES      GTSMATPHVS GVAALILQAH PSWTPDKVKT ----ALIETA DIVAPKEIAD
SUBTILISIN GTSMASPHVA GAAALILSKH PNWTNTQVRS ---SLENTT TKLGDS---

                660       670       680       690       700
PFUL      QKYTELDQGH GLVNVTKSWE ILKAINGTTL PIVDHWADKS YSDFAEYLGV
TCES      -----IAYGA GRVNVYKAIK YDDYAKLTFT GSVADKGSAT HTFDVSGATF
SUBTILISIN -----FYYGK GLINVQAAAQ *

                710       720       730       740       750
PFUL      DVIRGLYARN SIPDIVEWHI KYVGDTEYRT FEIYATEPWI KPFVSGSVIL
TCES      VTATLYWDTG SSDIDLYLYD PNGNEVDYSY TAYYGFEKVG YYNPTAGTWT

                760       770       780       790       800
PFUL      ENNTEFVLRV KYDVEGLEPG LYVGRIIIDD PTTPVIEDEI LNTIVIPEKF
TCES      VKVVSYKGAA NYQVDVVSDG SLSQSGGGNP NPNPNPNPTP TTDTQTFTGS

                810       820       830       840       850
PFUL      TPENNYTLTW YDINGPEMVT HHFFTVPEGV DVLYAMTTYW DYGLYRPDGM
TCES      VNDYWDTSDT FTMNVNSGAT KITGDLTFDT SYNDLDLYLY DPNGNLVDRS

                860       870       880       890       900
PFUL      FVFPYQLDYL PAAVSNPMPG NWELVWTGFN FAPLYESGFL VRIYGVEITP
TCES      TSSNSYEHVE YANPAPGTWT FLVYAYRTYG WADYQLKAVV YYG*

                910       920       930       940       950
PFUL      SVWYINRTYL DTNTEFSIEF NITNIYAPIN ATLIPIGLGT YNASVESVGD
```

Fig. 12(Cont'd)

```
                960       970       980       990      1000
PFUL GEFFIKGIEV PEGTAELKIR IGNPSVPNSD LDLYLYDSKG NLVALDGNPT

               1010      1020      1030      1040      1050
PFUL AEEEVVVEYP KPGVYSIVVH GYSVRDENGN PTTTTFDLVV QMTLDNGNIK

               1060      1070      1080      1090      1100
PFUL LDKDSIILGS NESVVVTANI TIDRDHPTGV YSGIIEIRDN EVYQDTNTSI

               1110      1120      1130      1140      1150
PFUL AKIPITLVID KADFAVGLTP AEGVLGEARN YTLIVKHALT LEPVPNATVI

               1160      1170      1180      1190      1200
PFUL IGNYTYLTDE NGTVTFTYAP TKLGSDEITV IVKKENFNTL EKTFQITVSE

               1210      1220      1230      1240      1250
PFUL PETTEEDINE PKLAMSSPEA NATIVSVEME SEGGVKKTVT VEITINGTAN

               1260      1270      1280      1290      1300
PFUL ETATIVVPVP KKAENIEVSG DHVISYSIEE GEYAKYVIIT VKFASPVTVT

               1310      1320      1330      1340      1350
PFUL VTYTTYAGPR VSILTLNFLG YSWYRLYSQK FDELYQKALE LGVDNETLAL

               1360      1370      1380      1390      1400
PFUL ALSYHEKAKE YYEKALELSE GNTIQYLGDI RLLPPLRQAY INEMKAVKIL

               1410
PFUL EKAIEELEGE E*
```

Fig. 13

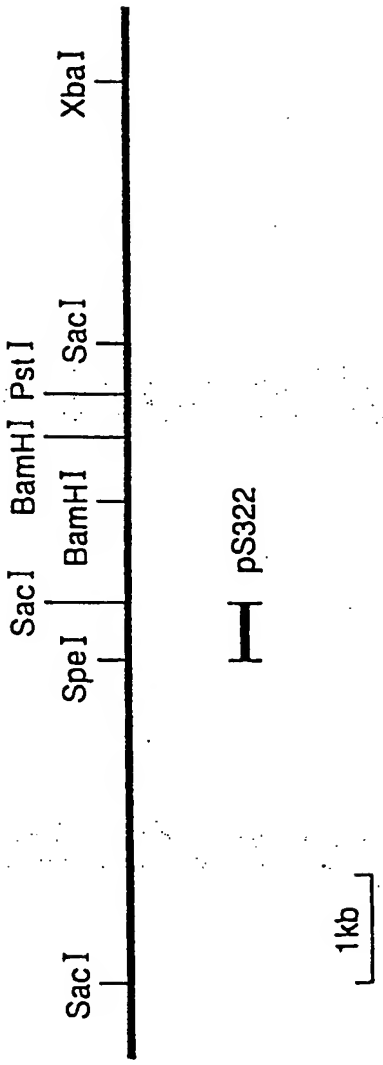
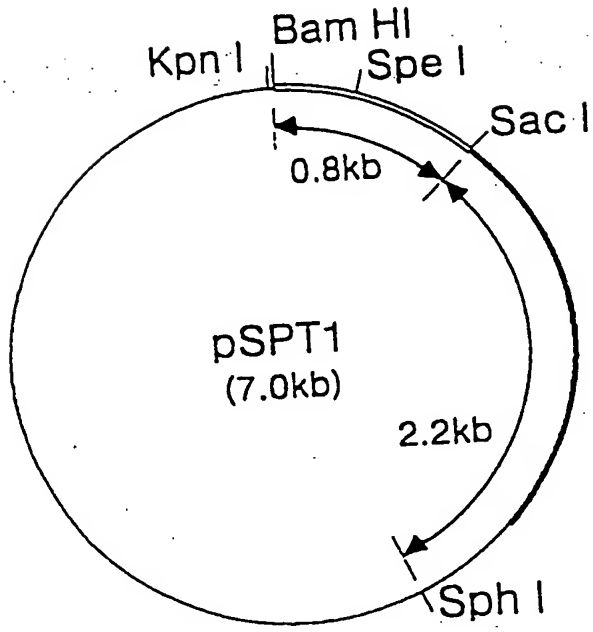


Fig. 14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Sph I

Fig. 15

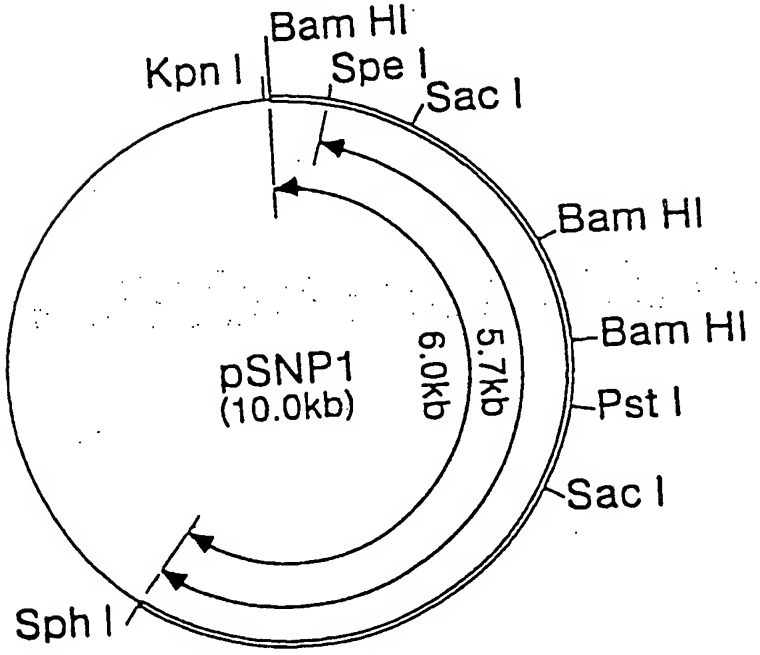

Bam HI
Kpn I
Spe I
Sac I
Bam HI
Bam HI
Pst I
Sac I
pSNP1
(10.0kb)
6.0kb
5.7kb
Sph I

Fig. 16

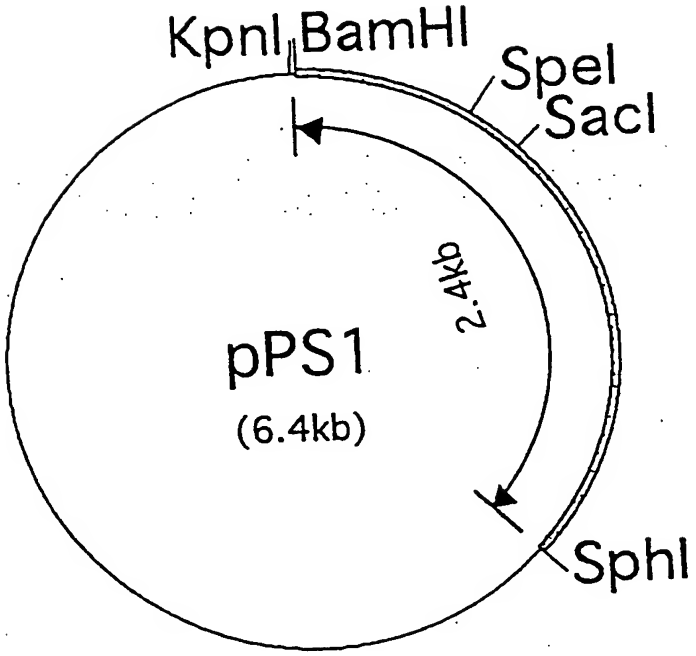

KpnI
BamHI
SpeI
SacI
2.4kb
pPS1
(6.4kb)
SphI

Fig. 17

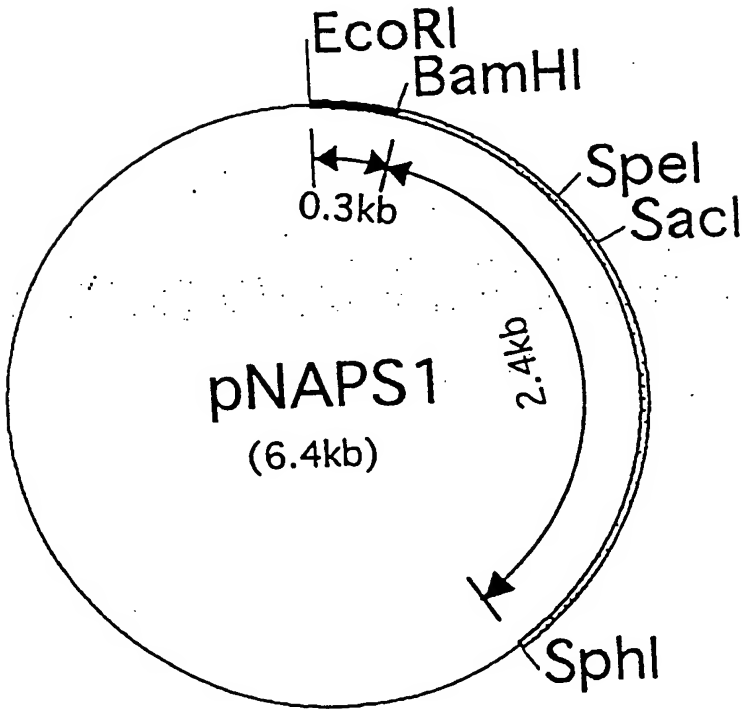

EcoRI
BamHI
0.3kb
SpeI
SacI
2.4kb
pNAPS1
(6.4kb)
SphI

Fig. 18

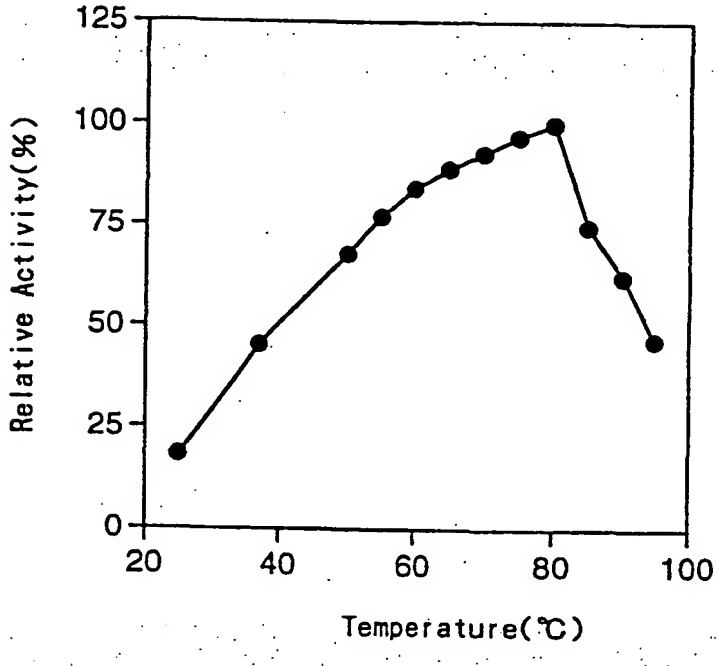

125
100
75
50
25
0
Relative Activity(%)
20
40
60
80
100
Temperature(℃)

Fig. 19

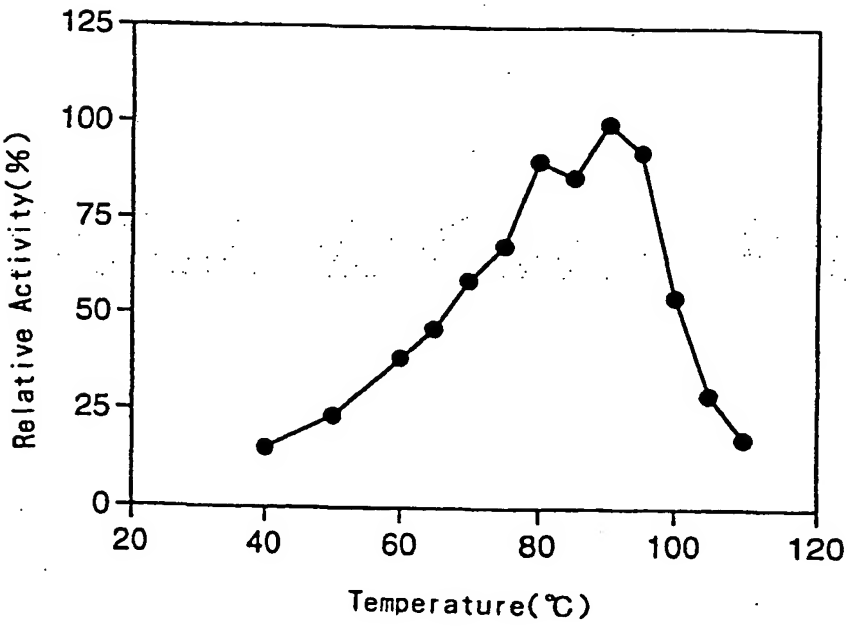

125
100
75
50
25
0
Relative Activity(%)
20
40
60
80
100
120
Temperature(℃)

Fig. 20

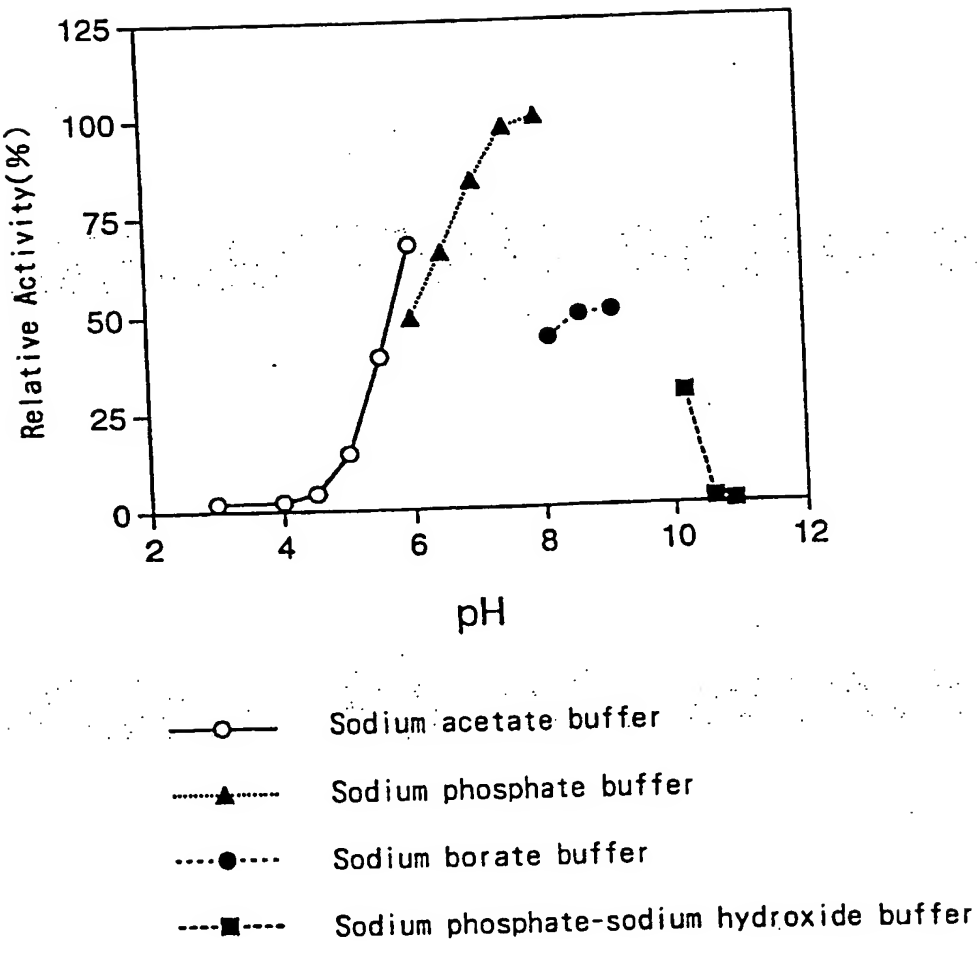

125
100
75
50
25
0
Relative Activity(%)
2
4
6
8
10
12
pH
Sodium acetate buffer
Sodium phosphate buffer
Sodium borate buffer
Sodium phosphate-sodium hydroxide buffer

Fig. 21

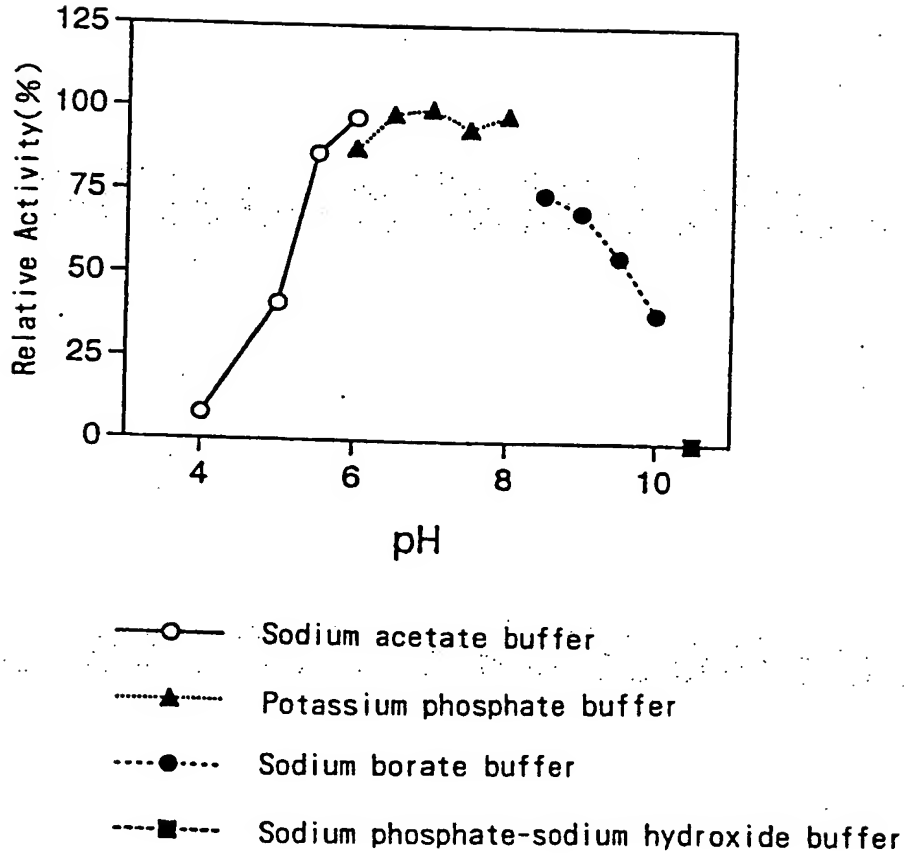

125
100
75
50
25
0
Relative Activity(%)
4
6
8
10
pH
Sodium acetate buffer
Potassium phosphate buffer
Sodium borate buffer
Sodium phosphate-sodium hydroxide buffer

Fig. 22

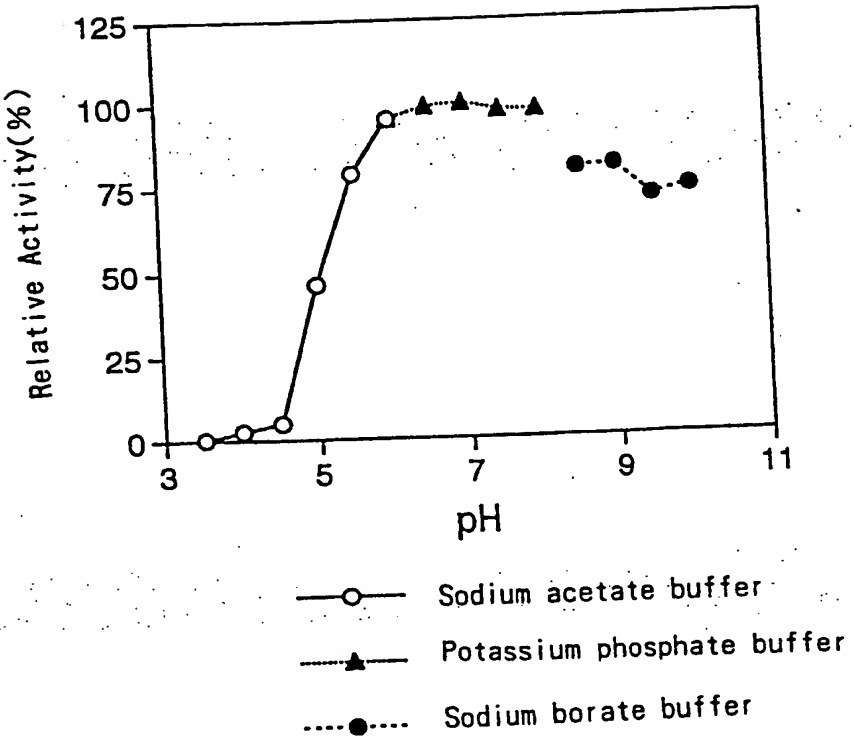

125
100
75
50
25
0
Relative Activity(%)
3
5
7
9
11
pH
Sodium acetate buffer
Potassium phosphate buffer
Sodium borate buffer

Fig. 23

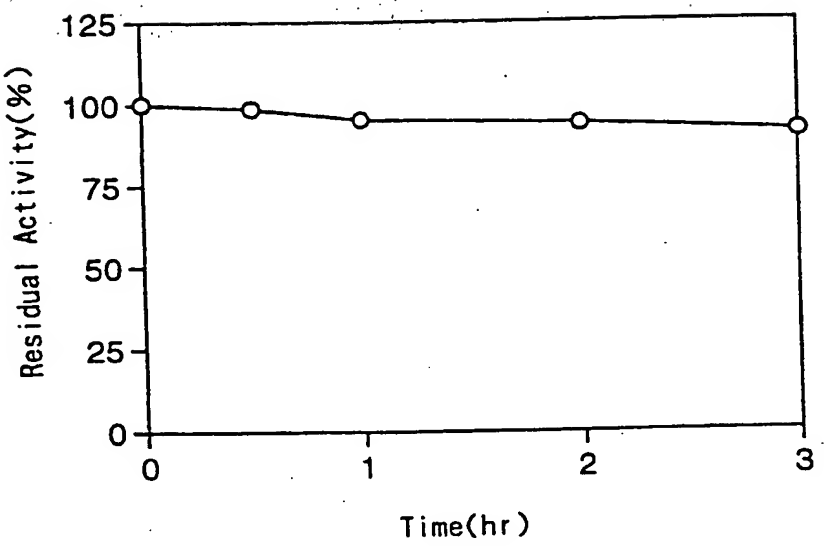

125
100
75
50
25
0
Residual Activity(%)
0
1
2
3
Time(hr)

Fig. 24

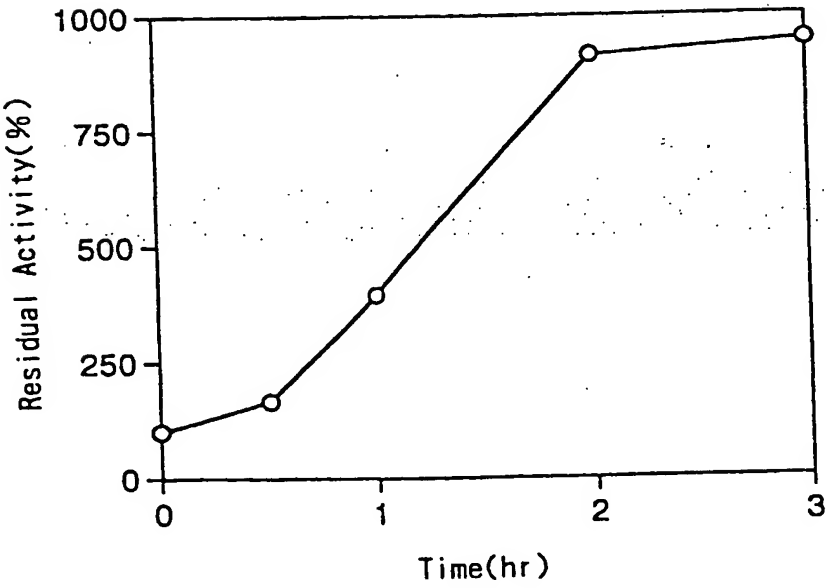

1000
750
500
250
0
Residual Activity(%)
0
1
2
3
Time(hr)

Fig. 25

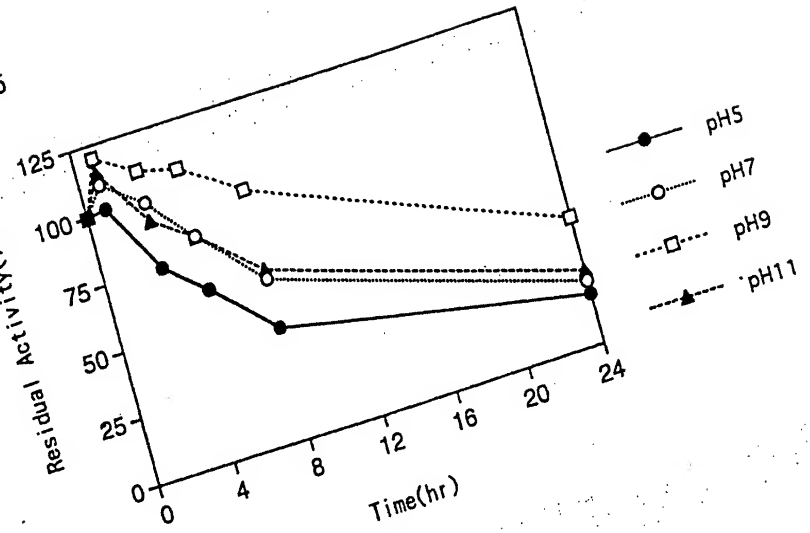

Residual Activity(%)
125
100
75
50
25
0
0
4
8
12
16
20
24
Time(hr)
pH5
pH7
pH9
pH11

Fig. 26

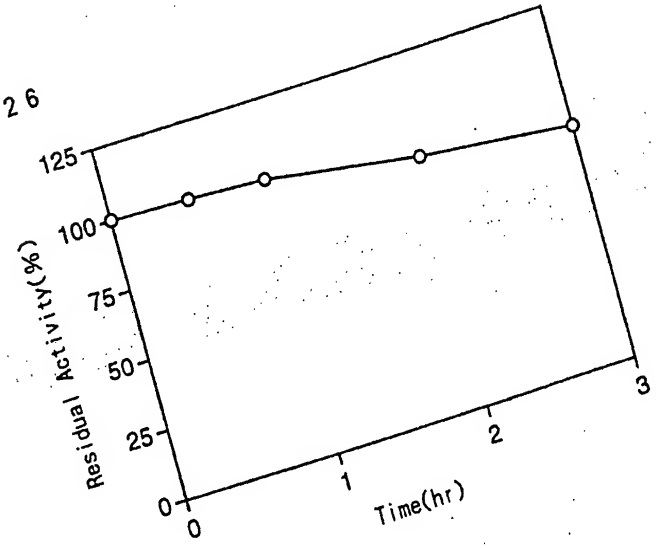

Residual Activity(%)
125
100
75
50
25
0
0
1
2
3
Time(hr)

Fig. 27

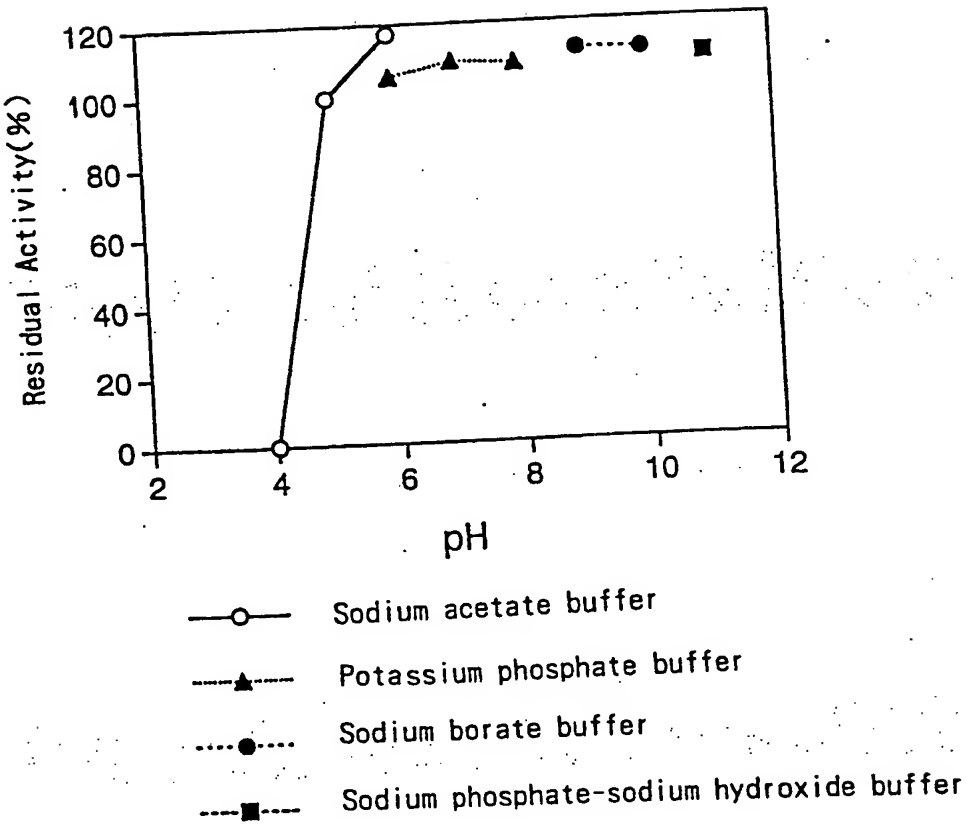

Residual Activity(%)
120
100
80
60
40
20
0
2
4
6
8
10
12
pH
Sodium acetate buffer
Potassium phosphate buffer
Sodium borate buffer
Sodium phosphate-sodium hydroxide buffer

Fig. 28

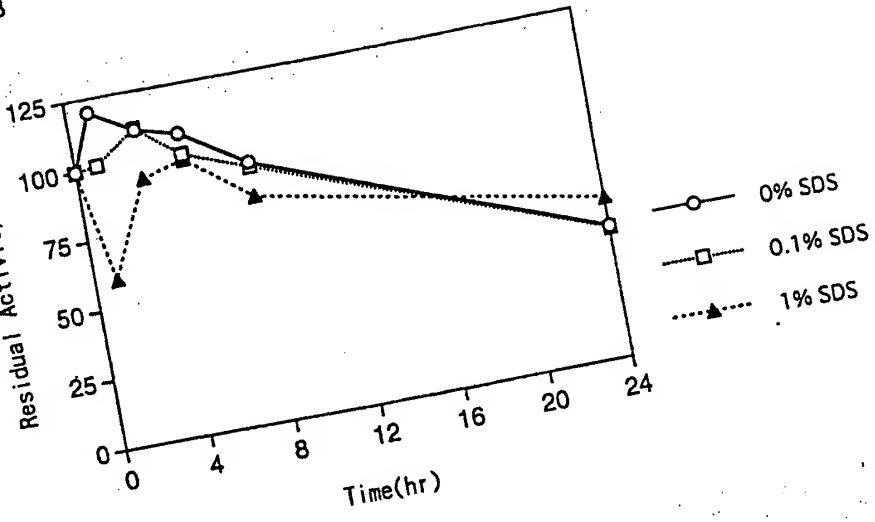

Residual Activity(%)
125
100
75
50
25
0
0
4
8
12
16
20
24
Time(hr)
0% SDS
0.1% SDS
1% SDS

Fig. 29

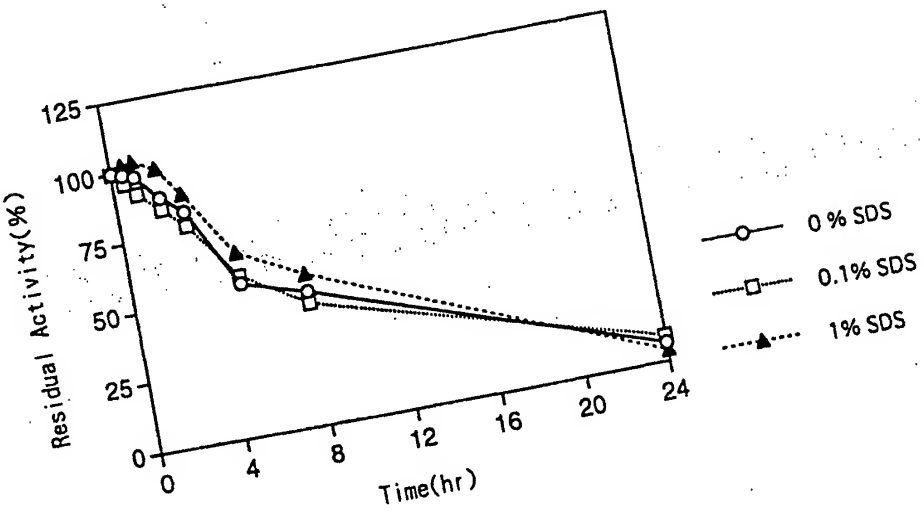

Residual Activity(%)
125
100
75
50
25
0
0
4
8
12
16
20
24
Time(hr)
0 % SDS
0.1% SDS
1% SDS

Fig. 30

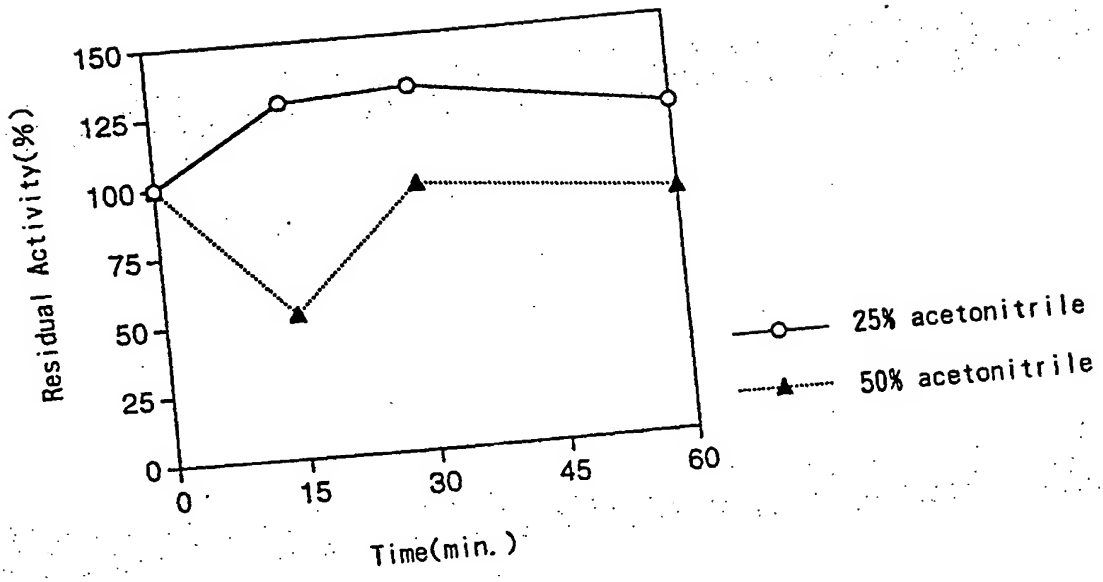

150
125
100
75
50
25
0
Residual Activity(%)
0
15
30
45
60
Time(min.)
25% acetonitrile
50% acetonitrile

Fig. 31

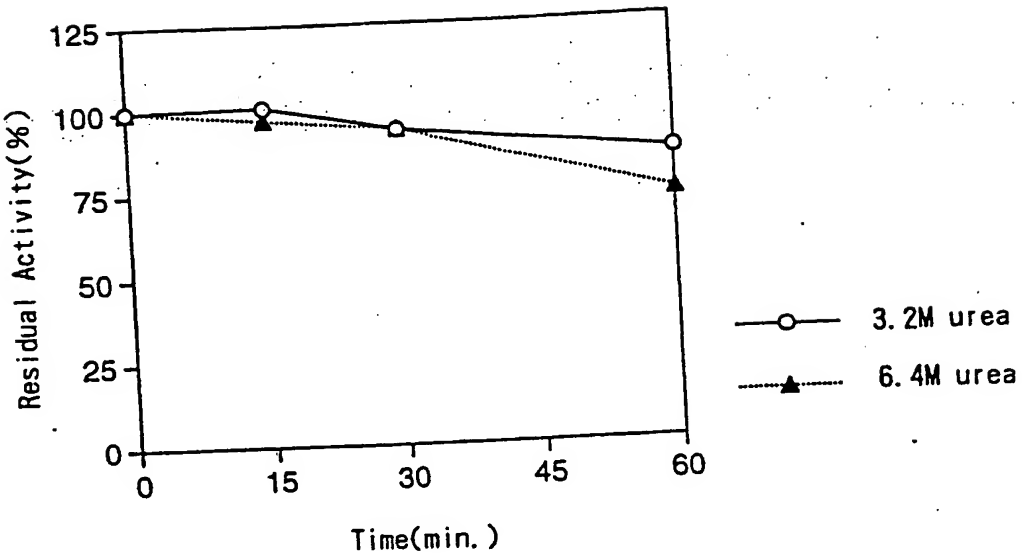
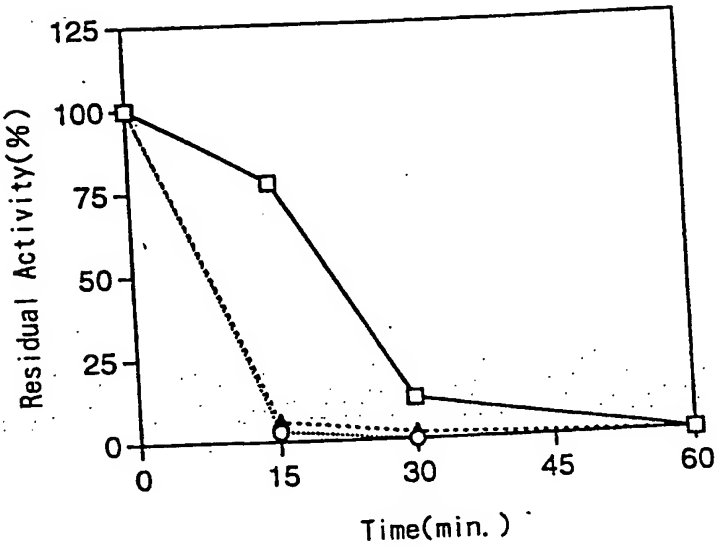

125
100
75
50
25
0
Residual Activity(%)
0
15
30
45
60
Time(min.)
3.2M urea
6.4M urea

Fig. 32


125
100
75
50
25
0
Residual Activity(%)
0
15
30
45
60
Time(min.)
1M guanidine hydrochloride
3.2M guanidine hydrochloride
6.4M guanidine hydrochloride

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP96/03253

A. CLASSIFICATION OF SUBJECT MATTER
Int. Cl^6 C12N15/57, C12N9/50

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int. Cl^6 C12N15/57, C12N9/50

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
BIOSIS PREVIEWS, CAS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JP, 7-184660, A (Takara Shuzo Co., Ltd.), July 25, 1995 (25. 07. 95)(Family: none)	1 - 4
A	JP, 6-197770, A (Takara shuzo Co., Ltd.), July 19, 1994 (19. 07. 94)(Family: none)	1 - 4
A	Michael Klingeberg et al. "Properties of extremely thermostable proteases from anaerobic hyperthermophilic bacteria" Appl. Microbiol. Biotechnol., 1991, Vol. 34, No. 6, p. 715-719	1, 2, 5, 6
A	WO, 91/19792, A1 (Nobo Norudisk A/S), December 26, 1991 (26. 12. 91) & JP, 5-507621, A & EP, 535110, A1	1, 2, 5, 6

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"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
January 29, 1997 (29. 01. 97)	February 12, 1997 (12. 02. 97)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)